Immunoglobulin lambda light chain gene rearrangements in human B-cell malignancies

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Immunoglobulin lambda light chain gene rearrangements in human B-cell malignancies

Immunoglobuline lambda lichte keten genherschikkingen in humane B-cel maligniteiten

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To the memory of my beloved sister, Hatice,
and her daughter, my dearest niece, Kadirye.

With love.

IMMUNOGLOBULIN LAMBDA LIGHT CHAIN GENE REARRANGEMENTS IN HUMAN B-CELL MALIGNANCIES

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Nature's mighty law is change (R.Burns)

CHAPTER 1

GENERAL INTRODUCTION ON NORMAL AND MALIGNANT HUMAN B-CELLS AND AIM OF THE STUDY

INTRODUCTION

Lymphocytes form the specific immune system, capable of recognizing and responding to any foreign antigen, while remaining indifferent to self components. Throughout human life, lymphocytes are continuously generated from pluripotent hematopoietic stem cells (1,2). These hematopoietic stem cells are already detectable in the yolk sac and in the fetal liver from the second month of gestation onwards (3-5). After birth, the hematopoietic stem cells are mainly found in the bone marrow (BM) (6).

Two types of lymphocytes exist: B-lymphocytes and T-lymphocytes. Progenitor B-cells differentiate into mature B-lymphocytes in the BM, while progenitor T-cells differentiate into mature T-lymphocytes in the thymus. Mature B- and T- lymphocytes recognize foreign antigens via surface receptor molecules, the so-called antigen specific receptors. The antigen specific receptors of B-and T-lymphocytes are called B-cell receptor (BCR) or immunoglobulin (Ig) molecules and T-cell receptor (TCR) molecules, respectively.

Differentiation of progenitor B-cells into mature B-lymphocytes is regulated via interaction with stromal cells in the BM (7-12). In the earliest stages, progenitor B-cells must be in direct contact with the stromal cells (10-12), and further differentiation is dependent on growth factors, such as cytokines and hematopoietic growth factors, which are secreted by the stroma (8,13). These growth factors and cytokines induce proliferation, differentiation, and maturation in an organized way (8,14,15).

IMMUNOPHENOTYPES OF B-CELLS DURING HUMAN B-CELL DIFFERENTIATION

The availability of monoclonal antibodies (McAb) reactive with functionally important and/or B-cell lineage-specific molecules has increased our understanding of developmental B-cell biology (Figure 1) (16-21). Application of these McAb for detailed immunophenotyping of BM cell samples allowed the recognition of different B-cell subpopulations, the estimation of their relative frequencies, and the design of hypothetical schemes of human B-cell differentiation, as illustrated in Figure 1 (20-22).

During B-cell differentiation all B-cells express the so-called pan-B-cell markers CD19, CD22, and CD72 (Figure 1). Besides these molecules, differentiation stage

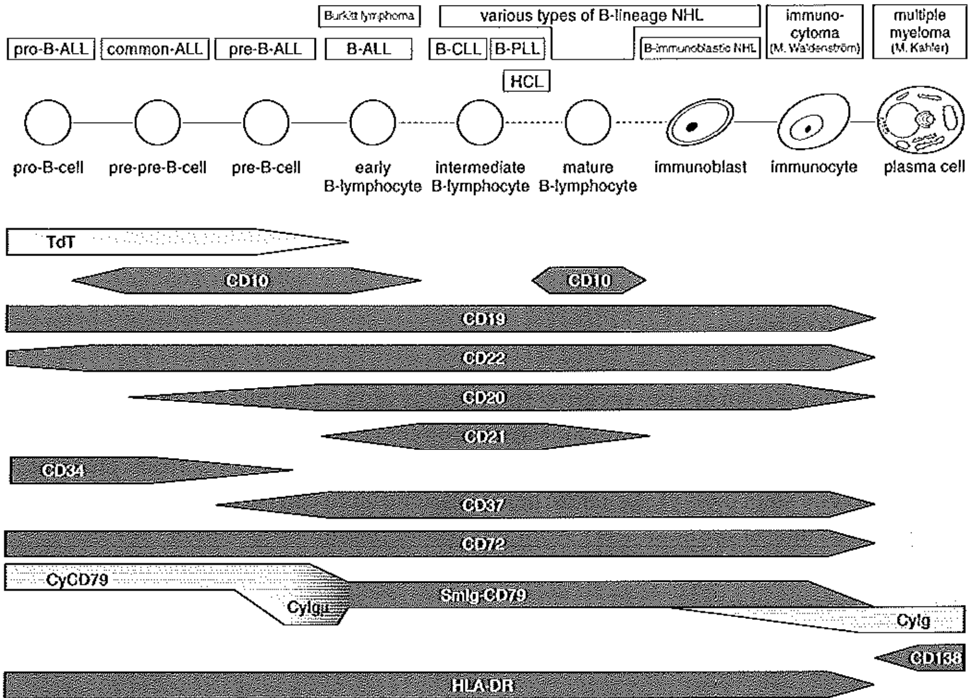

Figure 1. Schematic diagram of human B-cell differentiation from the putative pro-B-cell to the plasma cell. The expression of the various leukocyte antigens is indicated with shaded bars. The short upper bars indicate the various types of leukemias, lymphomas and multiple myeloma. Abbreviations: ALL = acute lymphoblastic leukemia, B-CLL = B-cell chronic lymphocytic leukemia, B-NHL = B-cell non-Hodgkin lymphoma, B-PLL = B-cell prolymphocytic leukemia, CyIg = cytoplasmic immunoglobulin, HCL = hairy cell leukemia, IgH = immunoglobulin heavy chain, SmIg = surface membrane immunoglobulin, TdT = terminal deoxynucleotidyl transferase (from: JJM van Dongen, ref. 181)

specific molecules are expressed. The CD34 molecule is only detected on precursor-B-cells: pro-B-cells, and pre-pre-B-cells. The nuclear enzyme terminal deoxynucleotidyl transferase (TdT) is present in pro-B-cells, pre-pre-B-cells, and pre-B-cells, while CD10 is only expressed in pre-pre-B-cells and pre-B-cells as well as on a part of mature B-lymphocytes, especially follicular B-lymphocytes. An additional differentiation marker is the expression of the CD79 molecule. In pro-B and pre-pre-B-cells CD79 molecules are detectable in the cytoplasm (CyCD79), whereas later on during differentiation these molecules are expressed on the cell surface in close association with surface membrane Ig molecules (SmIg). The CD20 and CD37 molecules are found in the more mature differentiation stages (Figure 1, Table 1)(51).

B-cell receptor

The antigen specific BCR or Ig molecules consist of two identical Ig heavy (IgH) and two identical Ig light (IgL) chains. The IgH chains are covalently linked to each

TABLE 1. Detailed information concerning clustered and non-clustered antibodies for immunophenotyping of normal and malignant human B-cells.

CD no.[a]	Antigen name(s)/function	mol. mass (kDa)	Reactivity with hematopoietic cells	Typical examples of McAb (no complete listing)[b]
Precursor markers				
CD34	precursor antigen	gp105-120	lymphoid and myeloid progenitor cells	HPCA-1/My10, HPCA-2/8G12, BI-3C5
CD117	SCFR (stem cell factor receptor); c-kit; SLF (Steel factor) receptor	gp145	hematopoietic progenitor cells, most colony forming cells, and mast cells	17F11, YB5.B8
-	TdT/function in Ig and TcR gene rearrangement (insertion of nucleotides at junction sites)	p58	immature lymphoid cells, small fraction of myeloid precursor cells, virtually all ALL, and some AML	conventional antisera and HTdT-6 McAb
B-cell markers				
CD10	common ALL antigen (CALLA)/ neutral endopeptidase (enke-phalinase)	gp100	subpopulation of precursor-B-cells, subpopulation of B-lymphocytes (follicular center cells), subpopulation of cortical thymocytes, granulocytes	J5, VIL-A1, BA-3
CD19	pan-B-cell antigen/function in B-cell activation; associates with CD21 antigen (CR2)	gp90	precursor B-cells and B-lymphocytes	Leu-12, B4, HD37
CD20	B-cell antigen/function in B-cell activation	p35	subpopulation of precursor-B-cells, all B-lymphocytes, follicular dendritic reticulum cells	- Leu-16, B1 - L26 detects intracellular epitope (CD20-Cy antibody)
CD21	B-cell antigen/CR2 (C3d receptor); EBV receptor	gp140	subpopulations of B-lymphocytes (e.g. follicular mantle cells), follicular dendritic reticulum cells, subset of thymocytes	OKB7, B2
CD22	B-cell antigen/function in B-cell adhesion and B-cell activation	gp135	precursor B-cells and B-lymphocytes	Leu-14/SHCL-1, RFB4, HD39
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)	gp45	FcεRIIa is expressed by a subpopulation of B-lymphocytes (e.g. follicular mantle cells) and B-CLL cells; FcεRIIb is expressed by subpopulation of B-lymphocytes, monocytes, eosinophils, dendritic cells	Leu-20/EBVCS-5, Tü1
CD37	B-cell antigen (tetraspan molecule)	gp40-52	B-lymphocytes; weak expression on T-cells, monocytes and granulocytes	RFB7, Y29/55

TABLE 1. **Detailed information concerning clustered and non-clustered antibodies for immunophenotyping of normal and malignant B-cells (continued).**

CD no.[a]	Antigen name(s)/function	mol. mass (kDa)	Reactivity with hematopoietic cells	Typical examples of McAb (no complete listing)[b]
CD72	B-cell antigen/ligand for CD5 antigen	gp43/39	precursor-B-cells and B-lymphocytes	J3-109
CD79a	mb-1; Igα (disulfide linked to CD79b and associated with SmIg)/signal transduction from SmIg to cytoplasm	gp32-33	precursor-B-cells (cytoplasmic expression; CyCD79a) and SmIg$^+$ B-cells (membrane expression; SmCD79a)	HM57 detects intracellular epitopes of CD79a (CD79a-Cy antibody)
CD79b	B29; Igβ (disulfide linked to CD79a and associated with SmIg)/signal transduction from SmIg to cytoplasm	gp37-39	precursor-B-cells (cytoplasmic expression; CyCD79b) and SmIg$^+$ B-cells (membrane expression; SmCD79b)	B29/123 detects intracellular epitope of CD79b (CD79b-Cy antibody)
CD138	plasma cell antigen	gp20	plasma cells and multiple myeloma	B-B4
-	mature B-cell antigen	gp105	B-lymphocytes	FMC7
-	pre-B CyIgμ (weak cytoplasmic expression of Igμ chain)	gp70	pre-B-cells; only μ heavy chains are weakly expressed in the cytoplasm (no mature Ig light chains)	selected anti-μ antisera
-	SmIg (surface membrane immuno-globulin); IgM, IgD, IgG, IgA, IgE	mol. mass is dependent on Ig class	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 immuno-globulin)	mol. mass is dependent on Ig class	CyIg positive cells (immunoblasts, immunocytes, and plasma cells)	conventional antisera and McAb
Non-lineage restricted markers				
CD5	T1 antigen/function in T-cell proliferation; ligand for CD72 antigen on B-lymphocytes	gp67	thymocytes and mature T-lymphocytes, subpopulation of B-lymphocytes; B-CLL	Leu-1, T1
CD6	T12 antigen/related to CD5 antigen	gp120	thymocytes and mature T-lymphocytes, subpopulation of B-lymphocytes; B-CLL	OKT17, T12

TABLE 1. Detailed information concerning clustered and non-clustered antibodies for immunophenotyping of normal and malignant B-cells (continued).

CD no.[a]	Antigen name(s)/function	mol. mass (kDa)	Reactivity with hematopoietic cells	Typical examples of McAb (no complete listing)[b]
CD11c	p150,95 antigen (integrin αX chain); associated with CD18 antigen/adhesion molecule; CR4 (C3bi, C3dg receptor)	gp150	monocytes, macrophages, granulocytes, subpopulations of lymphocytes (e.g. HCL-like cells in the spleen and NK-cells)	Leu-M5/SHCL3
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)	gp45	FcεRIIa is expressed by a subpopulation of B-lymphocytes (e.g. follicular mantle cells) and B-CLL cells; FcεRIIb is expressed by subpopulation of B-lymphocytes, monocytes, eosinophils, dendritic cells	Leu-20/EBVCS-5, Tü1
CD24	B-cell-granulocytic antigen; PI-linked protein on granulocytes	gp42	subpopulation of (precursor-) B-cells, granulocytes	BA-1, VIB-C5
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 common γ chain	gp55	activated T-cells, activated B-lymphocytes, activated macrophages; HCL	2A3, ACT-1
CD103	HML-1 (human mucosal lymphocyte 1 integrin); αE chain, which is associated with β7 chain	gp150,25	mucosa-associated T-lymphocytes (especially intraepithelial CD8$^+$ T-lymphocytes), 2-6% of blood lymphocytes; part of mucosal T-NHL (not other peripheral T-NHL) and HCL	B-ly7

a. CD = cluster of differentiation, as described during the Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989; Boston, 1993; Kobe, 1996).
b. Complete list of all relevant clustered and non-clustered antibodies can be obtained via J.J.M. van Dongen, Dept. of Immunology, Erasmus University Rotterdam, PO box 1738, 3000 DR Rotterdam, the Netherlands.

Abbreviations: ALL, acute lymphoblastic leukemia; AML, acute myeloid leukemia; CALLA, common ALL antigen; CyIg, cytoplasmic Ig; CLL, chronic lymphocytic leukemia; CR, complement receptor; EBV, Epstein Barr virus; FcγR, Fc receptor for IgG; FcεR, Fc receptor for IgE; FcμR, Fc receptor for IgM; GP, glycoprotein; gp, glycoprotein; HCL, hairy cell leukemia; HML, human mucosal lymphocyte; Ig, immunoglobulin; IL-2, interleukin 2; McAb, monoclonal antibody/antibodies; MHC, major histocompatibility complex; NK-cell, natural killer cell; R, reduced; SCFR, stem cell factor receptor; SLF, Steel factor; SmIg, surface membrane Ig. (from: JJM v Dongen and H Adriaansen, ref. 51)

other via disulfide bonds whereas the IgL chains are covalently bound to the IgH chains (Figure 2). The IgH and IgL chains consist of one variable domain, which is involved in antigen recognition, and one constant domain in case of IgL chains or three or four constant domains in case of IgH chains. The choice of IgH constant domains Cμ, Cδ, Cγ, Cε, or Cα determines the so-called Ig class: IgM, IgD, IgG, IgE, and IgA, respectively (23). Two types of IgL chains exist: Ig kappa (Igκ) and Ig lambda (Igλ).

Ig molecules are non-covalently associated with CD79a (mb-1) and CD79b (B29) molecules on the cell surface of B-lymphocytes; this so-called BCR complex plays a role in signal transduction upon antigen recognition (24-32). The B-lymphocytes can then mature to plasma cells which secrete Ig molecules, the so-called antibodies, which can recognize antigens.

Pre-B-cell receptor complex

In studies on murine precursor-B-cells an immature BCR was identified, the so-called pre-B-cell receptor (pre-BCR), which consists of Igμ proteins associated with λ5 and VpreB proteins, also called pseudo light chains (ψLC) (Figure 2) (33-40). λ5 and VpreB proteins have significant homology with the constant and variable domains of conventional Igλ light chains, respectively, but differ in that their encoding genes do not undergo rearrangements. Four human counterparts of the murine λ5 gene have been identified on chromosome 22: 14.1, 16.1, 16.2, and 18.1, of which especially the 14.1 protein is found to be covalently associated with Igμ (37, 38, 40-42). The pre-BCR is detectable on the cell surface during the pre-B-cell stage (40,41).

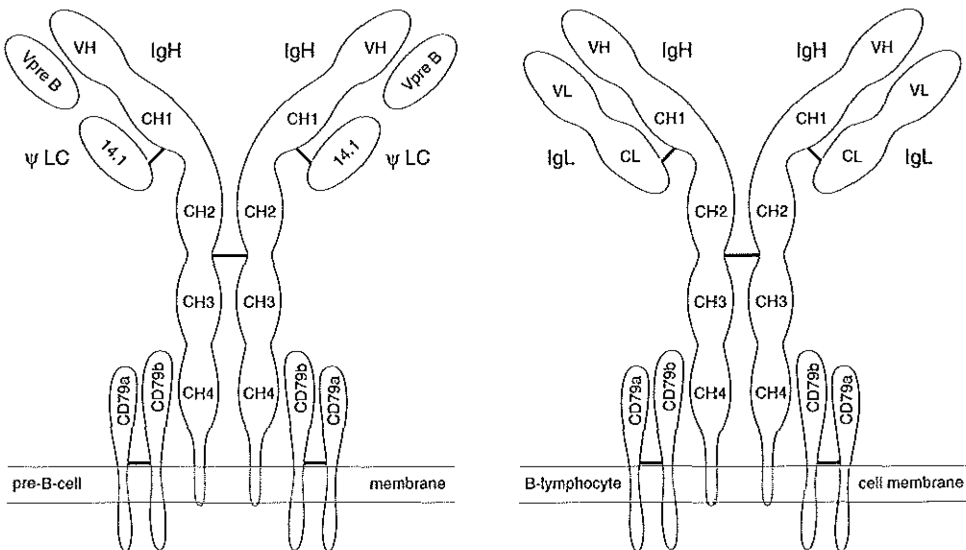

Figure 2. Schematic diagrams of the pre-BCR complex (left) and an IgM molecule (right), closely associated with CD79 chains on the cell membrane of a pre-B-cell and a B-lymphocyte, respectively.

It has been hypothesized that assembly of Igμ with ψLC is initiated by interaction of the VpreB protein with the variable domain of the Igμ chain. Probably the 14.1 protein can bind to the constant domain of the Igμ chain only if the VpreB protein and the variable domain of the IgH chain are assembled correctly. This complex associates with CD79 molecules and it is suggested that the pre-BCR interacts with the environmental stromal ligands in the BM to promote further B-cell differentiation. This interaction with BM stroma might trigger B-cell expansion and initiation of IgL chain gene rearrangements (see later) (43-48). Besides a permissive role for further differentiation and induction of rearrangements of IgL chain genes, it has been hypothesized that the pre-BCR complex might be involved in clonal selection via antigen or anti-idiotypic interaction (46-48).

IMMUNOPHENOTYPES OF B-CELL MALIGNANCIES

The various types of hematopoietic malignancies can be regarded as malignant counterparts of immature and mature hematopoietic cells (17,18). In Europe the overall incidence of these hematopoietic malignancies is 25 to 30 per 100,000 inhabitants and in The Netherlands (~15 x 10^6 inhabitants) ~4,000 new cases are diagnosed each year (49). Approximately 75% of all hematopoietic malignancies belong to the lymphoid differentiation lineage, representing acute lymphoblastic leukemias (ALL), chronic

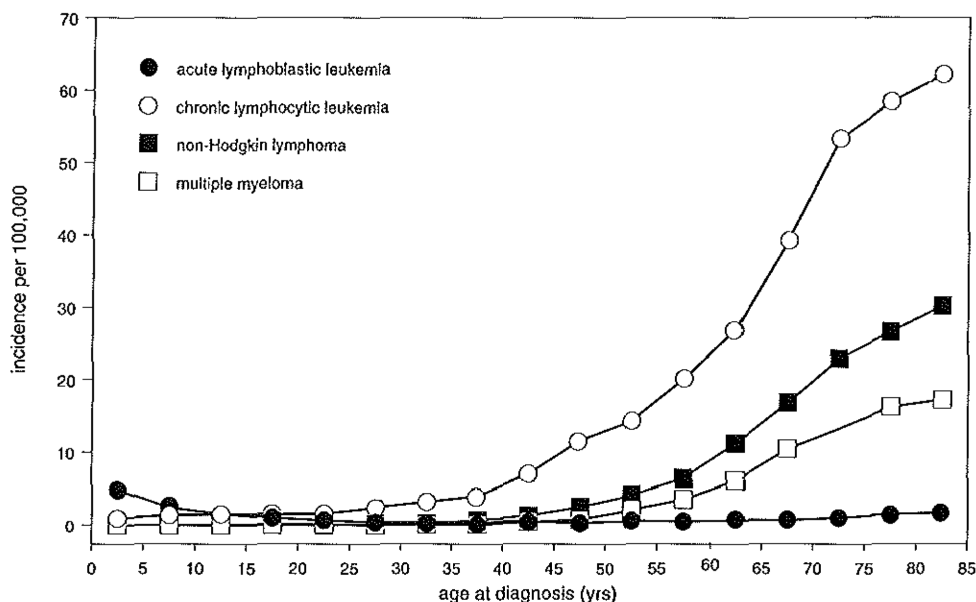

Figure 3. Age-related incidence of the four types of lymphoid malignancies in the Netherlands in 1989-1991. (from: Visser O, Coebergh JWW and Schouten LJ, ref. 49).

lymphocytic leukemias (CLL), non-Hodgkin lymphomas (NHL), and multiple myelomas (49). ALL has a relatively high incidence under the age of fifteen years and is regarded as a childhood leukemia, whereas CLL typically occurs in the elderly. Also the incidence rates of NHL and multiple myeloma increase with age (49). The age-specific incidence rates of these four types of lymphoid malignancies are given in Figure 3.

Most lymphoid malignancies belong to the B-lineage and represent malignant counterparts of cells in the various B-cell differentiation stages: 75-85% of ALL are precursor-B-ALL with phenotypes comparable to normal precursor-B-cells; ~95% of CLL and 90-95% of NHL belong to the B-lineage and resemble the various types of mature B-lymphocytes; multiple myelomas are malignant counterparts of plasma cells (Table 2). These various types of lymphoid malignancies can be recognized based on their cytomorphologic, histomorphologic, and immunophenotypic characteristics (Table 1) (50, 51).

TABLE 2. B-lineage and T-lineage origin of lymphoid malignancies.

	Acute lymphoblastic leukemia		Chronic lymphocytic leukemia	Non-Hodgkin lymphoma	Multiple myeloma
	childhood	adult			
B-lineage	80-85%	75-80%	95% (B-CLL, B-PLL, HCL)	90-95%	100%
T-lineage	15-20%	20-25%	5% (LGL, T-PLL, CTLL, ATLL[a])	5-10%	0%

a. In Japan and Caribian regions ATLL occurs in essential higher frequencies than in Europe and other Western countries.
Abbreviations: CLL, chronic lymphocytic leukemia; PLL, prolymphocytic leukemia; HCL, hairy cell leukemia; LGL, large granular lymphocyte leukemia; CTLL, cutaneous T-cell leukemia lymphoma; ATLL, adult T-cell leukemia lymphoma. (from: JJM van Dongen and HJ Adriaansen, ref. 51).

B-lineage ALL

Four main types of B-lineage ALL can be recognized (51,52). This concerns three types of precursor-B-ALL (pro-B-ALL, common ALL, and pre-B-ALL), which all express TdT and CyCD79 (31), whereas the rarely occurring B-ALL is negative for TdT but positive for the BCR complex (Figure 1). Virtually all B-lineage ALL are positive for the pan-B-cell markers CD19 and CD72 and generally also for CD22 (18,22,53). The CD10 antigen, the weak cytoplasmic expression of Igμ chains (CyIgμ), and the expression of SmIg are important markers for discrimination between the four subtypes of B-lineage ALL (Figure 1).

For the diagnosis of pre-B-ALL, weak expression of CyIgμ is a prerequisite: at least 10-20% of the ALL cells have to express this marker (52,54,55). Faint expression of the pre-BCR is seen on the cell surface of approximately 5% of pre-B-ALL (56).

Chronic B-cell leukemia

The vast majority of chronic B-cell leukemias express BCR molecules. Since a B-cell malignancy represents a clonal expansion of a single malignantly transformed B-cell, only one type of IgL chain is expressed. Therefore, the Igκ/Igλ distribution is useful for the detection of mature B-cell malignancies. Three main types of chronic B-cell leukemias are recognized: B-cell chronic lymphocytic leukemia (B-CLL), B-cell prolymphocytic leukemia (B-PLL), and hairy cell leukemia (HCL) (Figure 1, Table 3) (51,57).

B-CLL are characterized by the weak expression of SmIg molecules (58). However, in some B-CLL cases SmIg expression cannot be detected by use of fluorescence microscopy or flow cytometry. The most prevalent type of Ig class expressed is IgM, followed by double expression of IgM and IgD. An additional characteristic feature of B-CLL is the expression of the CD5 and CD6 antigens (50,57,58). Nevertheless a few B-CLL turned out to be negative for CD5. Most B-CLL are positive for the CD23 antigen, which is not present on the majority of other B-cell malignancies (58). Absence of the CD23 antigen is often associated with high levels of SmIgM expression, which suggests an intermediate stage between B-CLL and B-PLL (58).

B-PLL is a rare type of chronic B-cell leukemia. B-PLL cells show strong SmIg expression of IgM or co-expression of IgM and IgD (50,57). Generally, B-PLL cells are negative for CD5 and CD6, whereas the CD22 antigen is strongly expressed (57).

The immunophenotype of HCL cells is rather unique (50,57,59). They generally show strong SmIg expression, sometimes IgM or IgM/IgD double expression, but frequently IgG expression or IgG together with other (sub)classes; generally this concerns the IgG3 subclass (60). The expression of the CD20 and CD22 antigens is strong and the cells are generally positive for the CD11c, CD25 and CD103 antigens (50,59,61,62). The CD103 antigen turned out to be the most specific marker for HCL diagnosis, since only a small fraction of normal B-lymphocytes are positive for CD103 (20,61). Most HCL are negative for the CD24 antigen (57), whereas other chronic B-cell leukemias generally express the CD24 antigen (Table 3).

HCL-variant has a higher nucleus/cytoplasm ratio and the nucleus often contains a prominent nucleolus (63). The main immunophenotypic difference between HCL and HCL-variant concerns the lack of CD25 expression (α-chain of IL-2 receptor) on HCL-variant cells (62,63).

B-lineage NHL

NHL represent a heterogeneous group of solid neoplastic disorders which originate from cells of the immune system (64). In some types of B-lineage NHL involvement of blood and BM is frequently seen. Especially when the number of leukemic NHL-cells is high, discrimination between a chronic B-cell leukemia and a B-lineage NHL may be difficult (50,57,64).

Leukemic presentation of splenic lymphoma with villous lymphocytes (SLVL) is often misdiagnosed as B-CLL, B-PLL, or HCL. However, most SLVL are negative for

TABLE 3. Immunophenotypic characteristics of chronic B-cell leukemias, leukemic B-NHL, and multiple myeloma.

Markers	chronic B-cell leukemias				leukemic B-NHL			multiple myeloma
	B-CLL	B-PLL	HCL	HCLv	SLVL	MCL	FCL	
SmIg expression	++^{w}	++^{s}	++	++	++	++	++	–
CyIg expression	+/–	+/–	–	–	±	–	–	++
IgH isotype	μ,μδ,δ	μ,μδ	μ,μδ,γ,α	γ	μ,μδ,γ	μ,μδ,μγ	μ,μδ,γ	γ,α(δ,ε)
CD19	++	++	++	++	++	++^{w}	++	–
CD79	++	++	++	++	++	++	++	–
CD20	++^{w}	++	++^{s}	++	++	++^{s}	++	–
CD21	+	±	±	–	±	±	±	–
CD22	+^{w}	++^{s}	++^{s}	++	++^{s}	+	++	–
CD23	++	–	–	±	±	±	±	–
CD24	++	++	–	–	++	++	++	–
CD5/CD6	++	±	–	–	±	++	±	–
CD10	–	±	±	–	±	–	+	–
CD11c	+	–	++	+	+	–	–	–
CD25	±	–	++	–	±	–	–	–
CD103	–	–	++	+	±	–	–	–
CD138	–	–	–	–	–	–	–	++

Symbols: –, <10% of the leukemias is positive; ±, 10-25% of the leukemias is positive; +, 25-75% of the leukemias is positive; ++, >75% of the leukemias is positive; w, weak antigen expression; s, strong antigen expression.
Abbreviations: B-CLL: B-cell chronic lymphocytic leukemia; B-PLL: B-cell prolymphocytic leukemia; HCL: hairy cell leukemia; HCLv: HCL variant; SLVL: splenic lymphoma with villous lymphocytes; MCL: mantle cell lymphoma; FCL: follicular cell lymphoma. (from: JJM van Dongen and HJ Adriaansen, ref. 51).

the CD5 and CD103 antigens (Table 3) (51,65). Mantle cell lymphomas (MCL) are CD5^{+} and have a moderate to intense expression of SmIg molecules (66). In contrast to B-CLL, MCL strongly express the CD20 antigen and weakly express the CD19 antigen (66). Follicular cell lymphomas (FCL) do not exhibit a characteristic immunophenotype, although frequently expression of the CD10 antigen is seen (50,64).

Multiple myeloma

Multiple myeloma is a malignant neoplastic proliferation of plasma cells in the BM. Characteristically, multiple myeloma cells are negative for all pan-B-cell markers such as CD19, CD20, CD22, CD72, and CD79 (18,64). The most typical positive marker is the strong CyIg expression. Additional characteristics are the absence of the common leukocyte antigen CD45 and the presence of the CD38 antigen (18,64). Recently the CD138 antigen has been introduced as a valuable marker for identifying multiple myelomas (Table 3)(51).

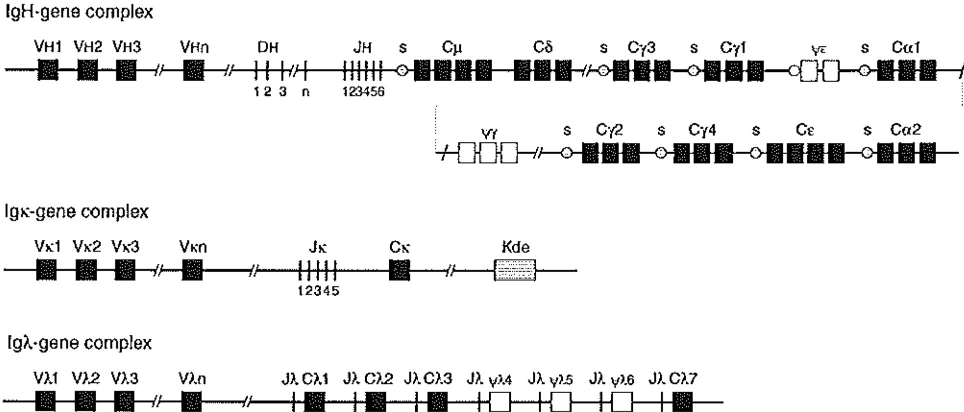

Figure 4. Schematic diagram of human Ig genes. The IgH gene complex consists of many (>100) V gene segments, at least 30 D gene segments, six functional J gene segments, and C gene segments for the constant domains of the various IgH classes and subclasses. Most C gene segments are preceded by a switch 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 or Jκ-Cκ gene regions 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 (from: JJM van Dongen and ILM Wolvers-Tettero, ref. 73).

Ig GENE REARRANGEMENTS DURING B-CELL DIFFERENTIATION

Like most genes in eukaryotic cells, the Ig genes consist of translated regions (exons), separated by intervening non-coding sequences (introns) (Figure 4). The variable domain of an IgH chain is encoded by an exon which consists of a combination of V (variable), D (diversity), and J (joining) gene segments (Figure 5), whereas a combination of V and J gene segments encodes the variable domain of an IgL chain (67-73).

During early B-cell differentiation, combinations are made of the available germline V, (D), and J gene segments of the Ig genes via a process called 'V(D)J recombination'. V(D)J recombination is mediated via sequence motifs flanking the germline gene segments, the so-called recombination signal sequences (RSS) (69-71,74-76). RSS are composed of a conserved palindromic heptamer and an AT-rich nonamer motif, separated by a spacer which consists of less conserved sequences (Figure 6) (77,78). These spacers are typically 12 or 23 bp long, and recombination occurs between two RSS with spacers of different length, the so-called 12/23 rule (79-82). V(D)J recombination is mediated by the regulated expression of several proteins, including the recombination activating genes (RAG), RAG1 and RAG2, and the so-called DNA-dependent protein kinase complex, which consists of three proteins: Ku-70, Ku-80, and a 350 kilodalton (kDa) catalytic subunit (p350) (83-88).

Initiation of the V(D)J recombination process occurs in two steps: in the first step, a single strand nick is introduced at the 5' end of the RSS heptamer. In the second step, this nick is converted into a hairpin structure on the coding side and a blunt end on the RSS side. Both nick and hairpin formation require an RSS, and the RAG1 and RAG2 proteins are both necessary and sufficient to carry out each step (82,89). The blunt RSS

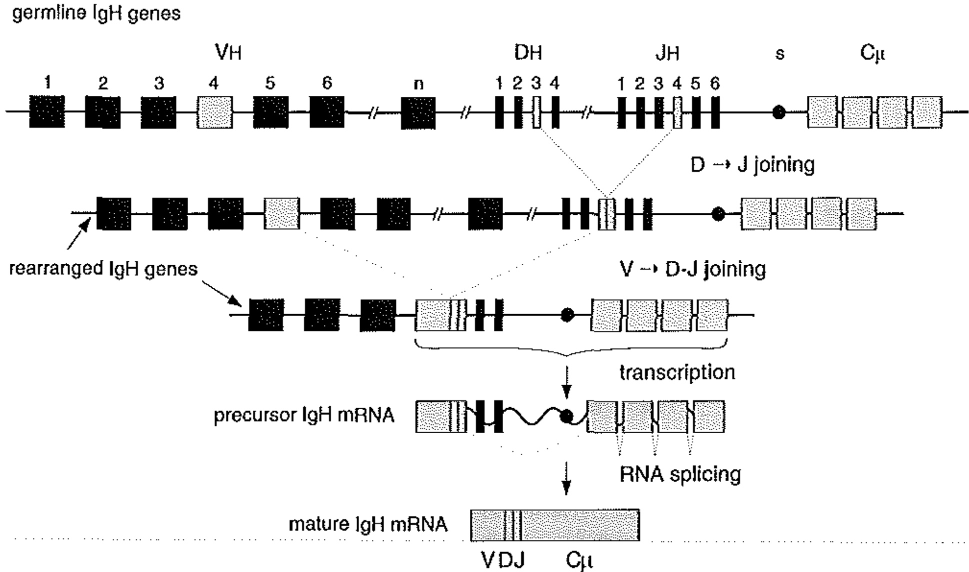

Figure 5. Schematic diagram of human IgH gene rearrangement. In this example first DH3 is joined to JH4, followed by VH4 to DH3-JH4 joining, thereby deleting all intervening sequences. The rearranged gene complex can be transcribed into precursor mRNA, which will be transformed into mature mRNA by splicing out all non-coding intervening sequences (from: JJM van Dongen and ILM Wolvers-Tettero, ref. 73).

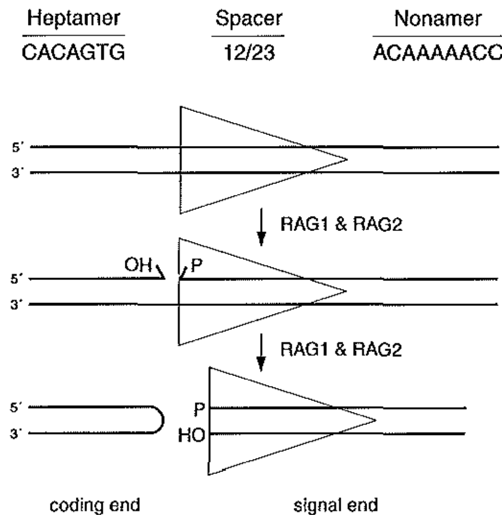

Figure 6. Model for the V(D)J cleavage reaction. In the first step, RAG1 and RAG2 proteins introduce a nick at the 5′ end of the signal sequence. The 3′-OH of this nicked signal is then coupled to the phosphate in the opposite strand, creating a coding end with a hairpin structure and a blunt, 5′-phosphorylated signal end. RAG1 as well as RAG2 are required for both steps (from: JF McBlane et al., ref. 89).

ends are joined to form 'signal joints' (Figure 6) (90). The opening of the hairpin structure and joining of the coding ends are thought to be mediated by the DNA binding proteins Ku-70 and Ku-80, and the p350 protein (85-88,90-92). The hairpin coding ends are opened prior to joining to another gene segment. During opening and joining of coding ends, deletion of germline nucleotides from the ends of the rearranging gene segments, short additions of template dependent self-complementary nucleotides (P nucleotides), and random insertion of the template independent nucleotides (N nucleotides) occur. These processes contribute to the antigen receptor diversity (93). Insertion of N nucleotides is mediated by the enzyme TdT (94).

Sequential Ig gene rearrangements during B-cell differentiation

Most of the information regarding early B-cell differentiation has been obtained from studies in mice. Several aspects of B-cell differentiation are also studied in man. Figure 7 summarizes the gene rearrangement events during B-cell development.

Ig gene rearrangements start at the pro-B-cell stage with rearrangement of a DH segment to a JH gene segment (Figures 5 and 7) (95,96). During the pre-B-cell stage, VH to DJH rearrangement occurs. As mentioned earlier, RAG1 and RAG2 proteins as well as TdT are crucial for these rearrangement processes. These proteins are highly expressed during the non-cycling pro-B and pre-B-cell stages (97-100). Once a productive IgH gene is formed on one of the two alleles, the IgH gene rearrangement process will stop (101-103). The produced IgH chains associate with both ψLC and CD79 chains (pre-BCR complex) and are expressed on the cell surface. The B-cells then enter the immature-B-cell stage, where IgL chain gene rearrangements occur (Figure 7). During IgL rearrangements, RAG1 and RAG2 expression is again upregulated. First, Igκ genes will start to rearrange (104-109). If Igκ gene rearrangements do not lead to production of a functional IgL chain, the Igλ light chain locus will start to rearrange.

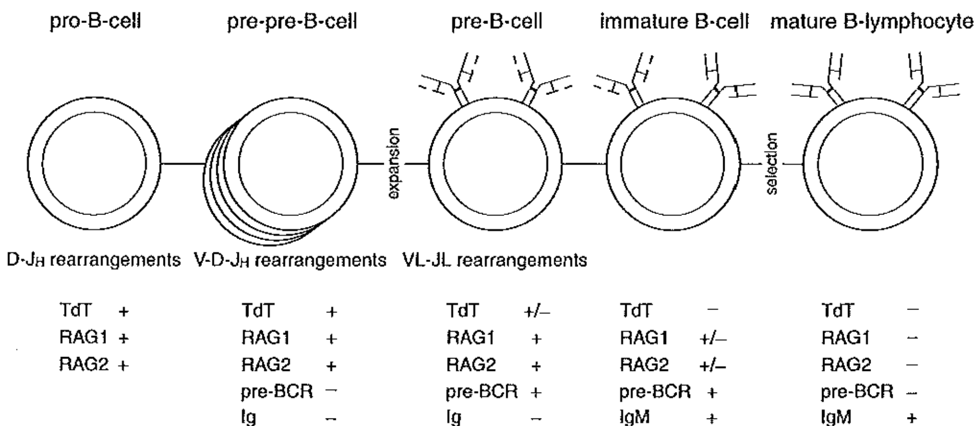

Figure 7. Model for early B-cell differentiation and the expression of several regulatory proteins (based on refs. 48, 97, 99, and 103).

Furthermore, it has been suggested that deletion of Igκ genes, mediated by the so-called kappa deleting element (Kde), might occur and that this plays an important role in the regulation of single IgL chain expression (110,111).

Receptor editing

If mature B-lymphocytes that entered the periphery are reactive to (soluble) autoantigens, they become 'anergic' (i.e. tolerant), which means that there is a block in the proximal part of the SmIg-mediated signalling pathway which results in the inability of the cells to become activated (112,113). However, earlier during B-cell differentiation immature B-cells might be able to avoid recognition of autoantigens by altering the antigen-binding regions of their SmIg molecules via a process called receptor editing (114-117).

Receptor editing can in principle be achieved by changing either IgH or IgL gene loci (114,117-119). Most of the VH gene segments have an embedded heptamer sequence upstream of their RSS, which is a potential recombination site for ongoing gene rearrangements (116). Replacements can occur through two pathways: firstly, an upstream VH gene segment replaces the V gene segment of an existing VHDHJH rearrangement, using the embedded heptamer sequence of the rearranged VH segment (48,116,120-122). Secondly, an existing D-JH complex on the second non-expressed allele can be replaced by rearrangement of an upstream DH gene segment to a downstream JH gene segment, followed by rearrangement of a VH gene segment to the 'newly' formed D-JH complex. It has been shown that proximity of rearranging gene segments is less important in the replacement processes of receptor editing, i.e. self-reactivity and subsequent receptor editing might induce rearrangements to far distant VH gene segments in order to achieve an efficient diversification of the IgH repertoire (116).

The junctional regions of the 'replaced' VHDHJH rearrangement have normal nucleotide deletions and N nucleotide insertions. The enzyme TdT, responsible for these N nucleotide insertions, is apparently reactivated during editing processes (123-126). Because of the short life span of immature B-cells, the ability of receptor editing remains limited. If no successful editing is achieved, the B-cells die via a process called programmed cell death.

Receptor editing can occur in IgL chain genes as well. It has been shown that due to the editing process the usage of the more upstream Vκ as well as the more downstream Jκ gene segments is increased (116-119,127). Furthermore, the relative frequency of Igλ usage increases, suggesting that Igκ gene rearrangements become 'overruled' by Igλ gene rearrangements.

Regulation of Ig rearrangement processes

Although comparable V(D)J recombination processes occur in both B- and T-cells, rearrangements of TCR genes are rarely seen in mature B-lymphocytes, and IgH genes show only some non-productive D-JH rearrangements in T-lymphocytes. During B-cell differentiation IgH rearrangements start before IgL rearrangements and Igκ genes

rearrange prior to Igλ genes. Apparently several mechanisms are responsible for the strict regulation of these rearrangement processes.

It has been suggested in several studies that rearrangements of Ig genes are preceded by transcription of particular gene segments, resulting in 'sterile' transcripts that do not encode a complete Ig protein. Transcription of germline Ig genes might 'open' the chromatine structure and thereby alter the accessibility of the locus for the recombinase enzyme complex. In this way sterile transcripts might 'guide' the Ig gene rearrangement process (95,112,128-132). It has been hypothesized that sequences within the Ig enhancers play an important role in the induction of sterile D-JH and VH transcripts (104,133-138).

The BCR itself plays an important role in the regulation of additional rearrangements. This is demonstrated in a transgenic mouse containing a functionally rearranged IgH transgene. Virtually all B-cells in this IgH transgenic mouse express the transgenic IgH chain. In these B-cells rearrangement of endogenous IgH genes is suppressed, but the endogenous IgL genes rearrange normally (139).

The expression of functional membrane bound pre-BCR molecules is thought to mediate allelic exclusion by turning off IgH gene rearrangements. Therefore, the last step of VH to DJH rearrangement on the second allele might not occur (101,102). Recently, Stanhope-Baker et al. (102) showed that Igμ protein expression has even a direct effect on the initiation of the Igκ gene rearrangements.

Further molecular diversification of Ig genes

Mature B-lymphocytes in the secondary lymphoid organs, e.g. lymph nodes and spleen, become activated upon recognition of antigens. These activated B-cells are found in the so-called germinal centers where they interact with follicular dendritic cells and T-cells; and where they further mature into memory B-cells or plasma cells. In germinal centers Ig genes undergo two additional modifications: somatic hypermutation and Ig class switch.

Somatic hypermutation

Somatic hypermutation is a tightly controlled process that occurs in germinal centers a few days after antigen-induced B-cell activation. The process of somatic hypermutation, also called affinity maturation, can increase the affinity of Ig to antigen 10 to 100 fold (140). The mutation rate of the somatic hypermutation process approaches 10^{-3} per base pair per generation, whereas the point mutation rate within the genome normally varies between 10^{-8} to 10^{-9} per base pair per generation (140-146). The mutations mainly concern point mutations of single nucleotides and rarely concern deletions or insertions (147). It has been suggested that one strand of the double helix is preferentially targeted for somatic hypermutation (148,149). Curiously, the hotspots for hypermutation are concentrated in the three so-called complementarity determining regions (CDR), which are known to interact with the antigen (150-153).

It has been shown that the distribution of somatic hypermutations is related to

transcription. The point mutation frequency was found to be increased approximately 150 base pairs downstream of the promoter start site, and the frequency then declines slowly over approximately 1500 base pairs (154-158). Furthermore, it has been suggested that the rate of somatic point mutations may be tied to the rate of transcription (159). Somatic hypermutation does not only occur in the expressed in-frame allele, but also in the non-expressed (out-of- frame) allele. Intron sequences around the V(D)J exon are also subject to somatic mutation, although they do not contribute to affinity selection (141,159,160). The rate of somatic mutation decreases during the life (161,162).

Immunoglobulin class switch

After antigen-induced activation, the B-lymphocytes proliferate and differentiate and also produce other Ig classes while retaining their BCR specificity: this mechanism enables the antibodies of a given specificity to change their effector function. The ability of an IgH variable domain to become associated with different CH domains is known as 'isotype switching' or 'class switching' (163-165). This process is mediated via so-called 'switch regions', which consist of G-rich tandemly repeated sequences of 1-10 kb in length. These switch regions are located upstream of each CH gene, except for the Cδ gene (164,166-169). The switch recombination process rearranges the Cμ switch region to another CH switch region, thereby deleting the intermediate DNA sequences. Sequential switching to further downstream CH genes may also occur (170). Ig class switch is induced via T-cell dependent antigen responses in conjunction with signals from T-cells and cytokines such as interleukin 4 (IL-4), IL-5, IL-10, and interferon γ.

IMMUNOGENOTYPES OF B-CELL MALIGNANCIES

Detection of clonal gene rearrangements

Similar to other neoplasms, B-cell malignancies are clonal diseases, which implies that the Ig gene rearrangements are identical in all cells of a certain B-cell malignancy. Clonal Ig gene rearrangements are detectable by Southern blotting and polymerase chain reaction (PCR) techniques. Southern blotting allows detection of deletion and relocation of gene segments based on changes in distances between cut-sites of restriction enzymes (73). PCR analysis allows the detection of joined gene segments in which the size of the PCR product is related to the position of the PCR primers and the size of the junctional region of the rearranged gene segments (171,172). This implies that clonality studies by Southern blotting take advantage of the combinatorial diversity (i.e. the relocation of gene segments), whereas clonality studies by PCR analysis are based on the junctional region diversity.

Immunogenotype of precursor-B-ALL

The vast majority of precursor-B-ALL (>95%) have IgH gene rearrangements, which is in line with the finding that IgH gene rearrangements start early during B-cell

differentiation. Also rearrangements in the Igκ gene complex are found in high frequencies (60%) (173,174).

Although the rearrangements in precursor-B-ALL seem to resemble rearrangements in normal B-cells, the continuous activity of the recombination system after malignant transformation induces several unusual rearrangements, such as high frequencies of TCR gene rearrangements. These so-called cross-lineage rearrangements of TCRβ, TCRγ, and TCRδ genes occur in 35%, 55%, and 90% of the precursor-B-ALL, respectively (173). Furthermore, in ~40% of precursor-B-ALL multiple rearranged IgH gene bands of different density were identified, indicating the occurrence of continuing rearrangement processes after malignant transformation resulting in subclone formation (174). Also replacements of the already existing D-JH rearrangement and/or VH replacements can occur (175-178). Most rearrangements in the Igκ gene complex in fact concern deletions of the Cκ or J-Cκ gene regions. These deletions are mediated via Kde rearrangements and occur in ~50% of precursor-B-ALL (179). The junctional regions of the deletional Igκ gene rearrangements in precursor-B-ALL contain N nucleotides (median: 4 to 5 nucleotides), which is in contrast to the virtual absence of N nucleotides in junctional regions of IgL genes in mature B-lymphocytes (Beishuizen et al., manuscript submitted). This might be explained by the fact that all precursor-B-ALL are positive for TdT and that the continuing gene rearrangements occur in the presence of TdT activity.

Immunogenotype of chronic B-cell leukemia, B-lineage NHL, and multiple myeloma

Virtually all chronic B-cell leukemias, B-lineage NHL, and multiple myelomas express Ig molecules with either Igκ or Igλ light chains. In man, 60% of the B-lymphocytes express Igκ and 40% express Igλ isotypes. Virtually all mature B-cell malignancies contain detectable IgH gene rearrangements, most of them on both alleles. All $Ig\kappa^+$ B-cell malignancies contain at least one rearranged Igκ allele, whereas Igλ gene rearrangements are rare (~5% of cases). Igλ gene rearrangements are detectable by Southern blotting in virtually all (>98%) $Ig\lambda^+$ B-cell malignancies; most of them have biallelic Igκ deletions (173,179,180). Cross-lineage TCR gene rearrangements are rare (<5%) in mature Ig^+ B-cell malignancies (173).

AIM OF THE STUDY

Much effort has been made to unravel Ig gene rearrangements during normal B-cell differentiation. Because of the complex structure of the Igλ gene locus, as compared to IgH and Igκ genes, most studies have focussed on the IgH and Igκ genes. Although 40% of B-lymphocytes express the Igλ chains, little is known about the rearrangements in the Igλ gene complex and the Igλ isotype usage during normal B-cell differentiation. Moreover, some B-cell malignancies cannot be correctly characterized based solely on IgH gene rearrangement analysis. In those cases additional information about IgL chain genes is needed. In $Ig\lambda^+$ B-cell malignancies, immunogenotyping of Igλ genes can provide the required information.

Analysis of Igλ gene rearrangements is, however, very complicated: firstly, the seven J-Cλ gene regions are highly homologous to each other; the J-Cλ2 and J-Cλ3 are even 98% homologous. Secondly, there is an alternative λ-like locus consisting of the 14.1, 16.1, 16.2, and 18.1 gene segments, which have >85% homology with the Cλ exons of the classical Igλ locus. Thirdly, sometimes a polymorphic region of 5.4 kb is present between the Cλ2 and Cλ3 exons which further complicates the analysis of the Igλ locus.

Therefore, the aim of our study was to develop tools for optimal and efficient analysis of rearranged Igλ genes. Information about IgL gene rearrangements might also give insight into the order of IgL gene re-arrangements as well as in the regulation of allelic exclusion.

Chapter 2 introduces the practical work: the protocols for DNA extraction, Southern blotting, and DNA probe labeling are described. **Chapters 3** and **4** describe the development of optimally chosen DNA probes and the careful selection of restriction enzymes for detection and identification of Igλ gene rearrangements in Igλ$^+$ B-cell malignancies. The Igλ 'isotype' rearrangement patterns of a large series of B-cell malignancies are described in **Chapter 5**. Finally, in **Chapter 6** we propose a rapid and efficient Southern blot strategy for Igλ gene studies, using only two or three probes in a few restriction enzyme digests. **Chapter 7** discusses the clinical relevance of Igλ gene rearrangement studies in the diagnosis of lymphoproliferative diseases. Furthermore, **Chapter 7** demonstrates how IgL gene studies in human B-cell malignancies can provide insight into the order of IgL gene rearrangements and the mechanism of allelic exclusion.

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2

Simplex sigillum veri (Boerhaave)
Eenvoud is het kenmerk van het ware

CHAPTER 2

IMMUNOGENOTYPING OF B-CELL MALIGNANCIES*

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INTRODUCTION

The rearrangement processes in immunoglobulin (Ig) genes start early during B-cell differentiation and mediate the coupling of variable (V), diversity (D), and joining (J) gene segments in case of Ig heavy (IgH) genes and the coupling of V and J gene segments in case of Ig light (IgL) genes. The many potential combinations of V, (D) and J gene segments form the basis of the so-called combinatorial diversity of Ig molecules, which is estimated to be $>5 \times 10^6$. This diversity is further extended by the so-called junctional diversity, which is based on the imprecise joining of the rearranged gene segments due to deletion and insertion of nucleotides at the junction sites (1).

Analogous to the occurrence of Ig gene rearrangements in normal immature and mature B-cells, the far majority of B-cell malignancies (>98%) also have rearranged Ig genes (2). Because B-cell malignancies are clonal diseases, the Ig gene rearrangements are in principle identical in all cells of a B-cell malignancy. Clonal Ig gene rearrangements are detectable by Southern blotting and by PCR techniques. Southern blot detection of clonal rearrangements is based on deletion and relocation of V, (D) and J gene segments, which result in changes in distances between cut sites of restriction enzymes. PCR detection of clonal rearrangements is based on the detection of coupled V-(D-)J gene segments, which are connected via the same (clonal) junctional region.

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According to our extensive experience over the last ten years, the Southern blot technique is highly reliable for detection of clonal Ig gene rearrangements, because false-negative and false-positive results can be prevented by the use of appropriate Southern blot protocols and optimal combinations of probes and restriction enzymes (2, 3, 4). Application of PCR techniques for detection of clonal Ig gene rearrangements is less reliable, because of the occurrence of false-positive and false-negative results. False-positive results might be due to difficulties in discrimination between polyclonal and monoclonal junctional regions in the obtained PCR products. False-negative PCR results might be caused by inefficient primer annealing due to somatic mutations in the rearranged V-(D-)J gene segments. Another cause of false-negative results is the occurrence of incomplete or unusual rearrangements, e.g. D-J rearrangements instead of V-D-J rearrangements, which require different primer sets for detection.

One should realize that the Southern blot technique is time-consuming and requires high molecular weight DNA, derived from fresh or frozen cell samples. In contrast, PCR techniques are rapid and allow the use of (partly) degraded DNA, e.g. derived from formaldehyde-fixed paraffin-embedded tissue samples. However reliable proof or exclusion of clonality (without false-positive and/or false-negative results) has major consequences for the diagnosis and management of patients with lymphoproliferative diseases. We therefore regard Southern blotting as the gold standard for *diagnostic* clonality studies in lymphoproliferative diseases.

SOUTHERN BLOTTING

For optimal Southern blot studies DNA is extracted from fresh or frozen blood, bone marrow and/or tissue samples (Protocol 1, page 54). The DNA samples are digested with restriction enzymes (Protocol 2, page 55). Restriction enzymes are endonucleases which reproducibly cut DNA only at sites where they recognize a specific nucleotide sequence, e.g. the restriction enzyme *Eco*RI recognizes the sequence GAATTC, whereas *Bgl*II recognizes the sequence AGATCT. The obtained DNA fragments (restriction fragments) are size-separated by agarose electrophoresis (Protocol 3, page 56). Subsequently, the restriction fragments are transferred (blotted) from the agarose gel onto a nitrocellulose or nylon membrane (Protocol 4, page 57). This membrane is incubated with a radiolabeled DNA probe, which hybridizes to complementary sequences of Ig genes (Protocol 5, page 58). Unbound probe is washed away and the location of the probe and thereby the size of the recognized restriction fragments can be detected by autoradiography. If appropriate restriction enzymes and DNA probes are used, the detected restriction fragments of rearranged Ig genes will differ from those of germline genes (1).

Figure 1 illustrates various aspects of Southern blot analysis of IgH genes: the germline restriction map of the JH-Cμ region with an appropriate JH probe (IGHJ6); the separation of restriction fragments in an agarose gel; and the autoradiographic results of hybridization with the radiolabeled IGHJ6 probe (1, 5).

In reactive polyclonal B cell proliferations many different Ig gene rearrangements are present, whereas in B cell malignancies clonal Ig gene rearrangements are found.

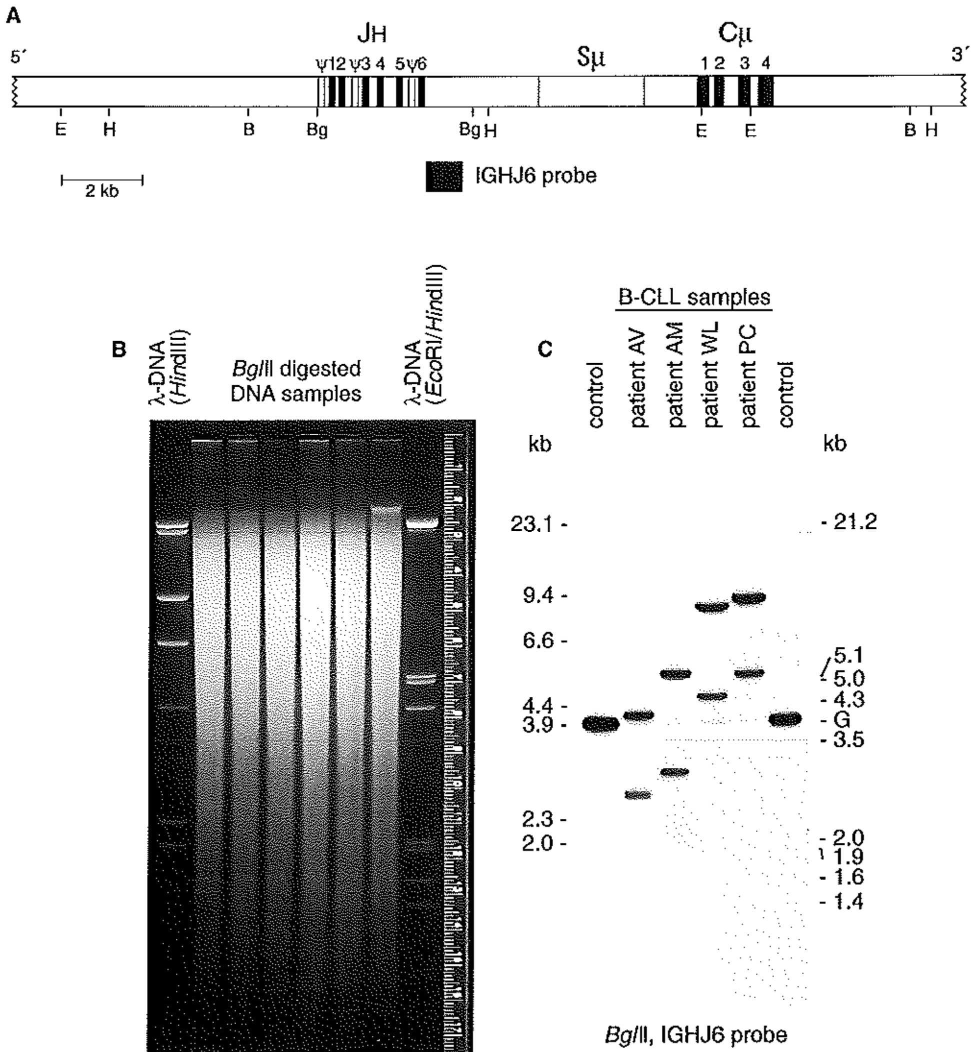

Figure 1. Southern blot analysis of IgH genes.

A. Restriction map of JH-Cμ region. The position of the relevant *Eco*RI (E), *Hin*dIII (H), *Bam*HI, and *Bgl*II (Bg) restriction sites are indicated. Also the location of the switch region (Sμ) is indicated. The solid bar represents the JH probe (IGHJ6).

B. Ethidiumbromide-stained agarose gel with size-separated *Bgl*II restriction fragments of control DNA and four different B-CLL DNA samples. The two outer lanes contain size markers (*left*: *Hin*dIII digested λ DNA; *right*: *Eco*RI/*Hin*dIII digested λ DNA). The DNA fragments were blotted to a nylon filter.

C. X-ray film after exposure to the nylon filter, which was hybridized to the ^{32}P-radiolabeled IGHJ6 probe. The size of the germline band (G) and the position of the size markers are indicated. The two control lanes contain the 3.9 kb germline band, whereas each of the four B-CLL lanes show two rearranged bands, due to biallelic IgH gene rearrangements.

Polyclonal rearrangements will not be detectable by Southern blotting, because the autoradiographic signals of single or a few restriction fragments are too weak to be visible within the background of many other restriction fragments. However, in case of a clonal cell population, many identical restriction fragments will comigrate in the agarose gel rendering their signals visible as a "rearranged band", which is different from the "germline band" (Figure 2). Two rearranged bands of comparable density will be visible if the clonal cell population has rearranged both alleles of the studied Ig gene (Figures 1 and 2). Thus, Southern blot analysis of Ig genes allows for discrimination between clonal rearrangements and polyclonal rearrangements.

Design of probes and choice of restriction enzymes

Southern blot analysis of Ig genes for diagnostic clonality studies requires well-designed probes and optimal probe/enzyme combinations in order to obtain reliable results.

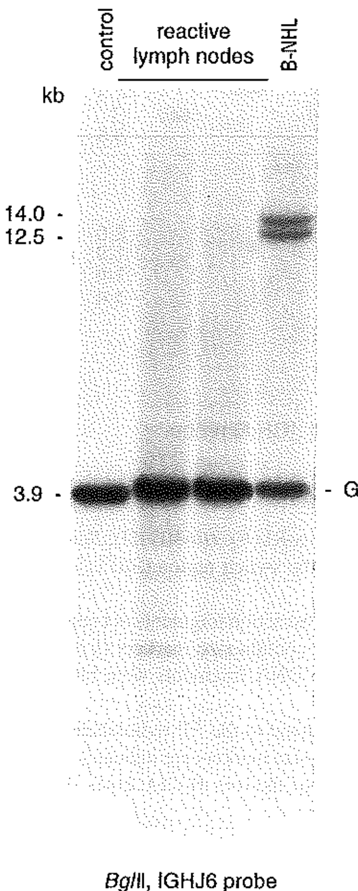

Figure 2. Southern blot analysis of IgH genes for discrimination between polyclonal and monoclonal B-cells. DNA from a germline control sample and from three lymph node biopsies of patients with suspect lymphadenopathy were digested with *Bgl*II and the Southern blot filter was hybridized with the IGHJ6 probe. The size of the germline band (G) and the rearranged bands are indicated in kb. In two lymph node biopsies no clonally rearranged bands were detectable; only a background of multiple faint non-germline bands were visible, which were derived from polyclonal (reactive) B-lymphocytes. In the third lymph node biopsy two rearranged bands were visible, indicating the presence of a clonal B-cell proliferation with biallelic IgH gene rearrangements.

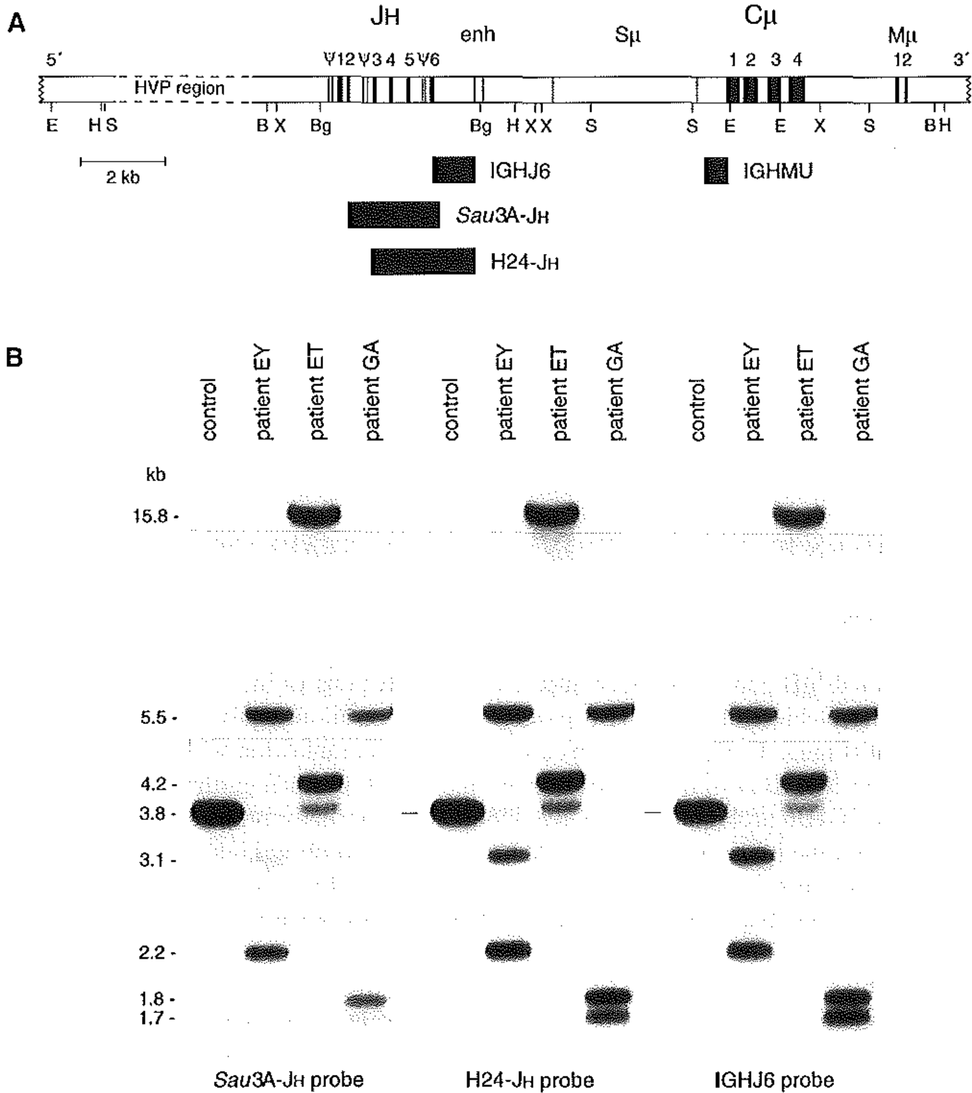

Figure 3. Comparison of three different JH probes for Southern blot analysis of IgH gene rearrangements.

A. Restriction map of 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 hypervariable polymorphic (HVP) region upstream of the JH region, the IgH enhancer (enh), the μ switch region (Sμ), and membrane μ (Mμ) gene segments are depicted. The solid bars represent the three JH probes and a Cμ probe: the IGHJ6, the *Sau*3A-JH, the H24-JH, and the IGHMU probe (5).

B. Comparison of three JH probes for Southern blot analysis of IgH genes in three precursor B-ALL patients at diagnosis. The *Bgl*II filter was successively hybridized with the *Sau*3A-JH probe, the H24-JH probe and the IGHJ6 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. Additional analyses revealed that these two rearranged bands represented JH6 rearrangements (5).

Optimally designed probes for detection of Ig gene rearrangements should fulfil the following criteria (1):

- the probes should not cross-hybridize to other genomic DNA fragments
- the size of the probes should be between 500 bp and 1 kb
- the probes should be positioned as close as possible to the rearrangement site
- if possible, J probes should be designed, because the majority of rearrangements in Ig genes involve J gene segments

For each probe optimally-chosen restriction enzymes should be used, which fulfil the following criteria (1):

- the germline restriction fragments should preferably be <10 kb in order to prevent comigration of germline and/or rearranged bands
- the restriction fragments should not be affected by genetic polymorphisms, such as restriction fragment length polymorphisms (RFLP)
- per probe at least two restriction enzyme digests should be used

SOUTHERN BLOT DETECTION OF IG GENE REARRANGEMENTS

IgH genes

Clonal IgH gene rearrangements, which involve one of the J gene segments, are easily detectable with the IGHJ6 probe, which is positioned just 3′ of the JH6 gene segment (Figure 1). Optimal results are obtained in combination with *Bgl*II digests or combined *Bam*HI/*Hin*dIII digests, because they result in small germline bands (5).

The IGHJ6 probe fulfils all above mentioned criteria for probe design. This is not the case for the frequently used *Sau*3A-JH and H24-JH probes (Figure 3), because these two probes recognize JH gene segment sequences, which might be deleted during rearrangement. Especially rearrangements to the JH6 gene segments will result in rearranged bands of lower density, which might be missed or misinterpreted as being caused by subclone formation (Figure 3) (5).

Sometimes a Cμ probe is used for detection of IgH gene rearrangements, but Cμ probes (e.g. IGHMU probe) are not optimal for detection of IgH gene rearrangements, because such probes need large restriction fragments to detect JH rearrangements (Figure 3). Nevertheless, the IGHMU probe might be useful for excluding IgH class switch in mature B-cell malignancies (5).

Igκ genes

The human Igκ locus contains five J gene segments and one C gene. Approximately 24 kb downstream of the Cκ gene the so-called kappa deleting element (Kde) is located (6, 7, 8). All functional Igκ gene rearrangements involve one of the five J gene segments and can easily be detected with a Jκ probe (IGKJ5 probe) or with a Cκ probe (IGKC probe) (Figure 4).

Approximately 50% of all precursor-B-acute lymphoblastic leukemias (ALL) and the vast majority of Igλ+ B-cell malignancies have Igκ gene deletions on one or both alleles.

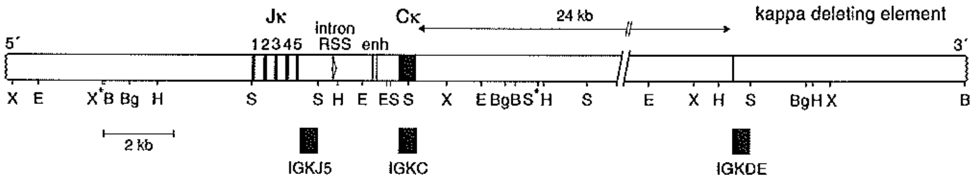

Figure 4. Restriction map of the human Igκ gene, i.e. the Jκ and Cκ region and Kde region, located ~24 kb downstream of the Cκ region. The location of relevant *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sac*I (S), and *Xba*I (X) restriction sites are indicated. Also the location of the intron RSS as well as the Igκ enhancer (enh) are depicted (9). 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).

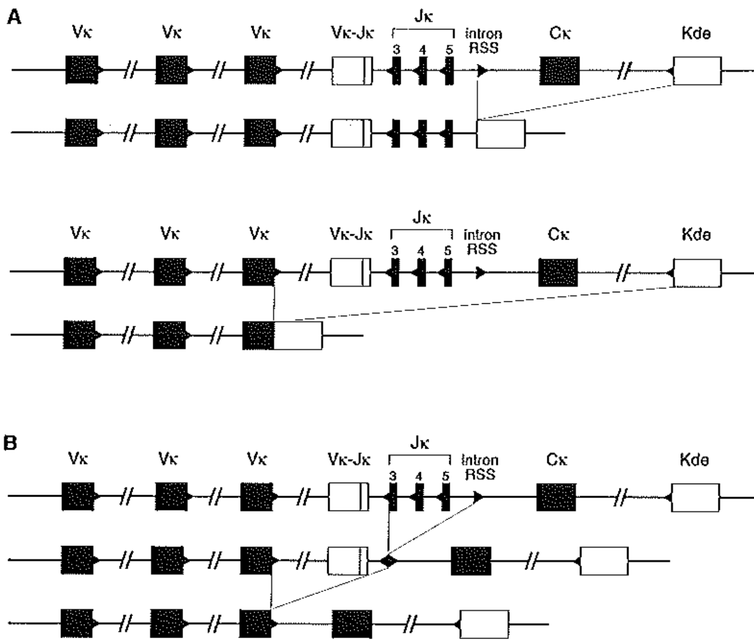

Figure 5. Schematic diagrams of Igκ gene deletions mediated by Kde rearrangements. Two types of Kde-mediated Igκ gene deletions can occur: Kde rearrangement either to the intron RSS (upper diagram) or to the RSS of a Vκ gene segment (lower diagram).

Even ~30% of Igκ⁺ B-cell malignancies have a monoallelic Igκ deletion with a functional Igκ gene rearrangement on the other allele (9). We demonstrated that >98% of all Igκ deletions are mediated via rearrangements of the Kde segment. Kde rearrangements can delete the Cκ gene (including the Igκ enhancer) or the complete Jκ-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 (Figure 5) (9). These Kde rearrangements can be identified precisely by use of the IGKDE probe (Figure 4).

Combined usage of the IGKJ5, IGKC, and IGKDE probes allows for detection and identification of virtually all Igκ gene rearrangements and deletions (Figure 6). Rearrangements

in the Jκ region are detectable with the IGKJ5 probe in *Sac*I, *Hin*dIII, *Eco*RI, *Bgl*II, or *Bam*HI digests (Figure 4). If no Cκ gene deletion has occurred, these rearrangements are also detectable with the IGKC probe in *Bam*HI or *Bgl*II digests, because their germline restriction fragments contain the complete Jκ-Cκ region (Figure 4). Kde-mediated Igκ gene deletions are detectable with the IGKDE probe in *Bgl*II, *Hin*dIII, or *Eco*RI digests (9).

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, which is not detectable with the IGKJ5 probe (Figures 4 and 6). The third type of Igκ gene deletion, (i.e. deletion of Jκ gene segments without deletion of the Cκ gene segment) is rare (<2% of all Igκ deletions) and can be detected by successive

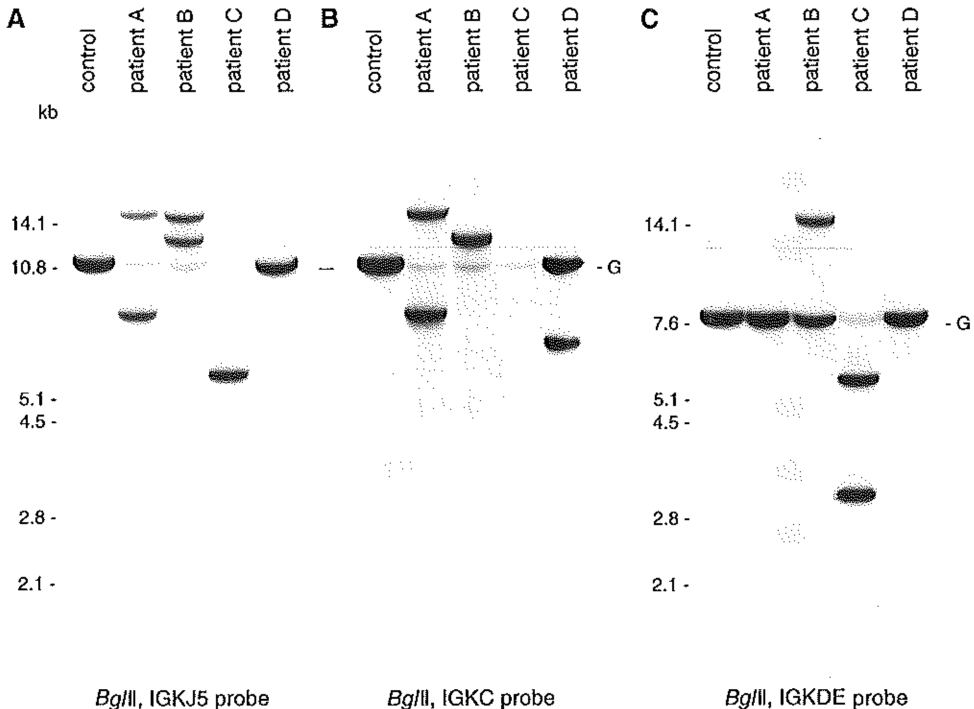

Figure 6. Southern blot analysis of four patients, who were selected for their Igκ gene rearrangement and/or deletion patterns. Control DNA and DNA from three chronic B-cell leukemias samples (lane two, three, and four) and one precursor B-ALL (lane five) were digested with *Bgl*II. The DNA filter was 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 (9).

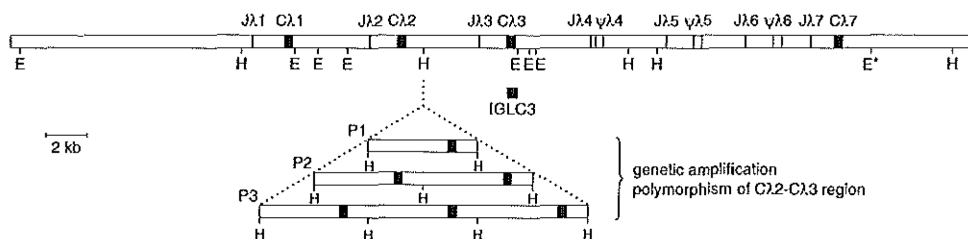

Figure 7. Organization of the J and C gene segments of the human Igλ gene complex, including the genetic amplification polymorphism of J-Cλ2/J-Cλ3 gene region. The location of the relevant *Eco*RI (E) and *Hin*dIII (H) restriction sites are indicated (1). The solid boxes represent functional Cλ exons and dotted boxes are non-functional (pseudo; ψ) Cλ exons. The location of the IGLC3 probe is indicated as a solid bar; this probe recognizes all Cλ exons of the classical Igλ gene complex and the surrogate λ-like gene complex (15).

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 (Figure 6) (9).

Igλ genes

The classical human Igλ locus contains seven Cλ gene segments, each preceded by a J gene segment (Figure 7) (10, 11). The J-Cλ1, J-Cλ2, J-Cλ3 and J-Cλ7 regions are functional and code for the four distinct Igλ isotypes, whereas J-Cλ4, J-Cλ5 and J-Cλ6 regions are non-functional due to deletions and/or insertions in the Cλ gene segments (12, 13, 14). The seven J-Cλ regions are homologous; this especially concerns the J-Cλ2 and J-Cλ3 gene regions with a homology of 98% (11, 13).

Whereas the J gene segments of the IgH and Igκ locus are clustered in small regions of ~2.5 kb and 1.4 kb, respectively (Figures 1 and 4), the seven Jλ gene segments are scattered over a region of ~30 kb (Figure 7). Adequate Southern blot analysis would need multiple Jλ probes to cover this large region, which is laborious and time-consuming. Therefore, a single Cλ probe (e.g. the IGLC3 probe) is generally used, which recognizes all Cλ exons due to the high homology of ~85% (1, 13). This approach is hampered by four limitations. Firstly, for detection of J gene rearrangements with a Cλ probe only restriction enzymes without cut sites in the seven J-Cλ introns can be used, such as *Eco*RI and *Hin*dIII (Figure 7). Secondly, the Cλ probe does not only recognize Cλ exons of the classical Igλ locus, but also cross-hybridizes to Cλ exons of the surrogate λ-like gene complex, 14.1, 16.1, 16.2, and 18.2 (16, 17). Thirdly, rearranged bands might comigrate with one of the multiple Cλ germline bands, especially in case of large rearranged and large germline fragments; finally, a genetic amplification polymorphism in the Cλ2-Cλ3 gene region causes extra bands, which make the interpretation of the Southern blots even more complicated (Figure 7) (15).

Traditionally, Cλ probes are used in *Eco*RI digests, but this frequently leads to false-negative results, because the multiple germline and rearranged bands in *Eco*RI digest are rather large (Figure 8). Our studies in a large series of Igλ$^+$ B-cell malignancies demonstrated that *Eco*RI/*Hin*dIII double digests allowed for detection of ~95% of

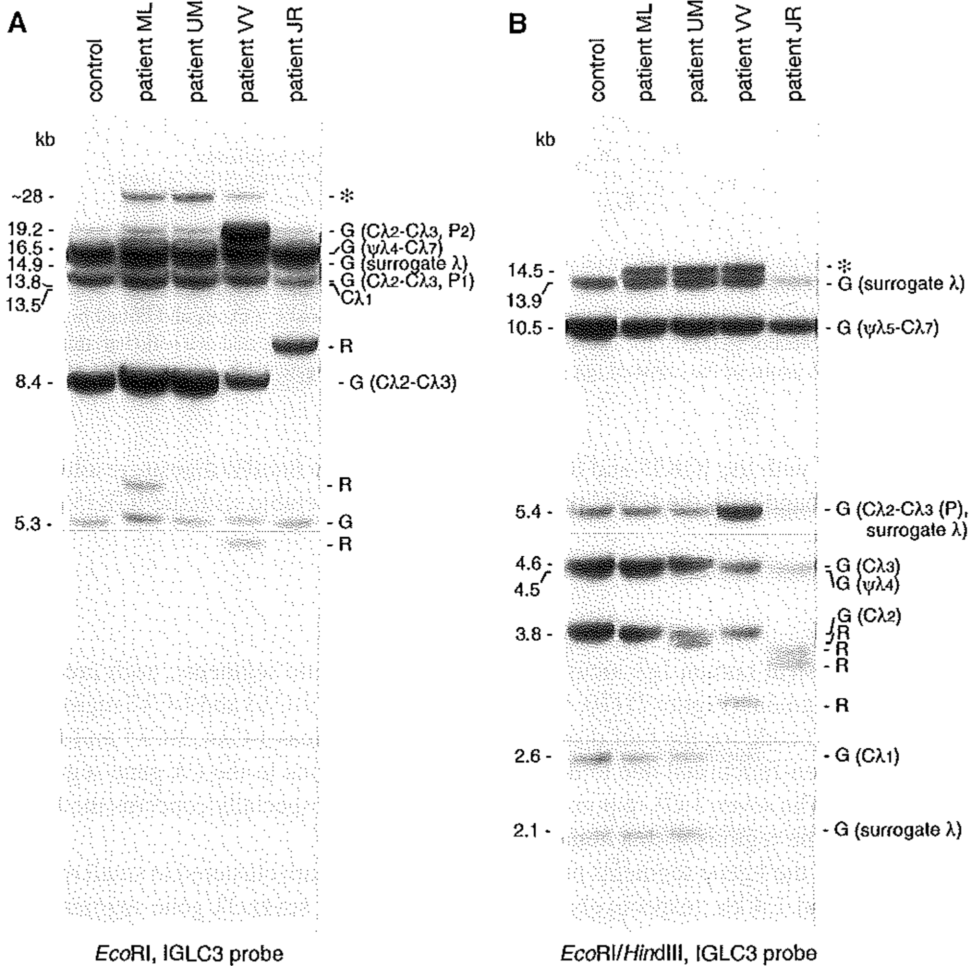

Figure 8. Southern blot analysis of Igλ genes in Igλ⁺ B-cell malignancies. The DNA samples were digested with *Eco*RI (A), and *Eco*RI/*Hin*dIII (B), size-fractionated, blotted onto a nylon membrane and hybridized with the IGLC3 probe. Lane 1 contains a control DNA sample and shows the germline (G) positions. Lane 2 contains DNA with a clonal Igλ gene rearrangement, which was detectable in the *Eco*RI digest, but exceptionally not in the combined *Eco*RI/*Hin*dIII digest. Lanes 3, 4, and 5 contain DNA with Igλ gene rearrangements which are detectable with combined *Eco*RI/*Hin*dIII digests (15).

all Igλ gene rearrangements, whereas *Eco*RI digests only detected ~80% of Igλ gene rearrangements (Figure 8) (1, 15).

According to our experience the IGLC3 probe in *Eco*RI/*Hin*dIII digest is a quite effective single probe/enzyme combination for detecting Igλ gene rearrangements. Nevertheless still ~5% of all Igλ gene rearrangements will be missed, probably due to comigration of the rearranged bands with one of the multiple germline bands, which represent the Cλ gene segments of the classical and surrogate Igλ loci. This problem can only be solved by designing Jλ probes for each J gene segment.

PITFALLS AND LIMITATIONS OF SOUTHERN BLOTTING

Reliable Southern blot analysis of Ig genes is only possible if sufficient knowledge concerning potential pitfalls and limitations is available. Major problems in the interpretation of Southern blots are caused by usage of inappropriate probes, too large restriction fragments, occurrence of restriction fragment length polymorphisms (RFLP), and partial digestion of DNA (1).

Inappropriate probes

Optimal probes should be designed according to the criteria, indicated in the top of page 44. Major problems are caused by J probes which overlap with clustered J gene segments (e.g. *Sau*3A-JH probe in Figure 3). Such probes result in weak rearranged bands of different density, because they recognize sequences which are deleted in case of J gene rearrangements. This leads to several problems:

- The relative size of the clonal cell population will be underestimated.
- False-negative results will be obtained, because the rearranged band can be missed. For example a clonal JH6 rearrangement will be missed with the *Sau*3A-JH probe, if the clonal cell population is <50% (5).
- Differences in density of rearranged bands will be erroneously interpreted as being caused by subclone formation (Figure 3).

Inappropriate probe/enzyme combinations

For each probe appropriate restriction enzymes should be selected resulting in germline and rearranged restriction fragments, which should preferably be less than 10 kb. The larger the size, the more chance of comigration of the rearranged and/or germline bands (Figure 9).

Table 1 summarizes the optimal restriction enzymes for each Ig gene probe. Also the size (in kb) of each germline restriction fragment is given.

Occurrence of restriction fragment length polymorphisms

RFLP cause additional bands, which might be misinterpreted as rearranged bands. Thus, for each probe/enzyme combination it should be carefully evaluated whether RFLP occur. For this purpose at least 50 healthy individuals should be studied in order to evaluate the allelic frequency of potential RFLP (see Table 1). Nevertheless, rare RFLP still can cause interpretation problems. The chance of such problems can drastically be reduced by using at least two different restriction enzymes per probe and by evaluating whether the density of the "rearranged" band is comparable to the density of the germline band (1).

Special attention is needed for the hypervariable polymorphic (HVP) region, upstream of the JH gene segments (Figure 3). When the IGHJ6 or other JH probes are used, this HVP region causes RFLP in *Eco*RI and *Hin*dIII digests in 80% of individuals

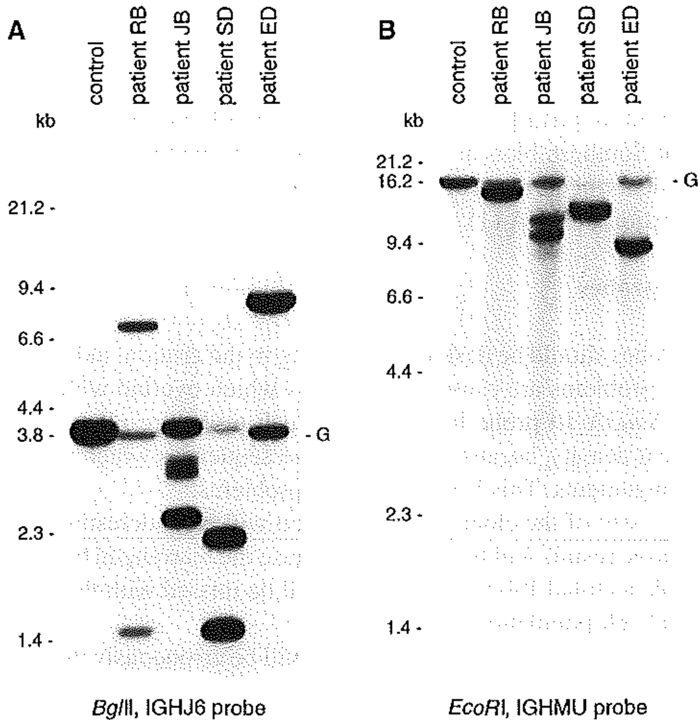

Figure 9. Southern blot analysis of IgH genes in four representative precursor B-ALL at diagnosis. Control DNA and DNA from precursor-B-ALL samples were digested with (A) *Bgl*II and (B) *Eco*RI. The *Bgl*II filter was hybridized with the IGHJ6 probe and the *Eco*RI filter with the IGHMU probe. In the *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 the rearranged bands was obtained in the *Bgl*II filter.

(18, 19, 20). The IGHJ6 probe should therefore in principle not be used in combination with *Eco*RI and/or *Hin*dIII restriction enzymes.

Partial digestion of DNA

Some restriction sites might appear to be resistant to digestion. Partial digestion might cause major problems for correct interpretation of Southern blot results. According to our experience partial digestion rarely occurs for the probe/enzyme combinations given in Table 1, except for *Eco*RI digests in Igλ gene studies. The *Eco*RI site downstream of the Cλ7 exon (Figure 7), appears to be resistant to digestion (15). When using the IGLC3 probe, partial digestion results in an extra band of ~14.5 kb in *Eco*RI/*Hin*dIII and ~28 kb in *Eco*RI digests (Figure 8).

TABLE 1. DNA probes and restriction enzymes for detection of clonal Ig gene rearrangements.

Ig complex	DNA probe[a]	Restriction enzyme	Restriction fragment (kb)[b]	Allelic frequencies of RFLP	
IgH gene	IGHJ6	*Bgl*II	3.8 (6.7)	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)
		*Bam*HI	16.0	0%	(0/150)
	IGHMU	*Bam*HI	16.0	0%	(0/150)
		*Eco*RI	16.2	HVP region	
Igκ gene	IGKJ5	*Sac*I	1.9	0%	(0/150)
		*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	*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	*Sac*I	11.4	0%	(0/114)
		*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)
Igλ gene	IGLC3	*Eco*RI/*Hin*dIII[c]	2.6 (λ1) 3.8 (λ2) 4.6 (λ3) 4.5 (ψλ4) 10.5 (ψλ5-λ7)	0% (except for 5.4 kb band in case of polymorphic Cλ2-Cλ3 amplification)	(0/112)
		*Eco*RI[c]	13.5 (λ1) 8.4 (λ2 and λ3) 16.5 (ψλ4-λ7)	0% (except for 13.8 kb, 19.2 kb, and 24.6 kb bands in case of polymorphic Cλ2-Cλ3 amplification)	(0/56)

a. The position of the DNA probes is given in Figures 3, 4 and 7.
b. The numbers in parentheses represent the sizes of the polymorphic germline restriction fragments.
c. Only the germline bands of the classical Igλ locus are given. The IGLC3 probe also hybridizes to Cλ gene segments of the surrogate Igλ genes (see Figure 8).

Detection limit of Southern blot technique

According to our extensive experience the detection limit of the Southern blot technique is ~5% (5 clonal cells between 100 normal cells). Some investigators claim that they routinely can detect 1% or even 0.2% clonal cells. However, in our opinion this is a misrepresentation of the sensitivity in routine practice (1).

Furthermore, one should be aware that the sensitivity will also be influenced by the background of normal (non-clonal) rearrangements. If many reactive polyclonal B-lymphocytes are present (see Figure 2), the detection limit might be as high as 10%.

CONCLUSION

So far, Southern blot analysis of Ig genes is the most reliable technique for diagnostic clonality studies in patients, suspected to have a B-cell malignancy. Southern blot probes should be designed according to strict criteria and should be combined with restriction enzymes, which do not result in RFLP. If possible, two different restriction enzymes per probe should be used.

Optimal probe/enzyme combinations for detection of clonal IgH gene rearrangements are the IGHJ6 probe in *Bgl*II and *Bam*HI/*Hin*dIII digests; for Igκ gene rearrangements and deletions: IGKJ5, IGKC, and IGKDE probes in *Bgl*II and *Hin*dIII (or *Bam*HI/*Hin*dIII) digests; and for Igλ gene rearrangements: IGLC3 in *Eco*RI/*Hin*dIII digests.

It is clear that the Southern blot technique remains the gold standard for diagnostic clonality studies. Nevertheless, is worth studying to what extent the time-consuming Southern blot technique can be replaced reliably by rapid PCR analyses.

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Protocol 1. Extraction of DNA

Use either mononuclear cell (MNC) fraction after ficoll density centrifugation of peripheral blood or bone marrow, or use tissue biopsy samples. Particular care should be taken to avoid contamination of phage or plasmid DNA to overcome many troubles and misinterpretations.

1. Dissolve cells/tissue biopsy in TNE buffer (10 mmol/l Tris-HCl, pH 7.6; 100 mmol/l NaCl; 10 mmol/l EDTA) at an estimated concentration of about 5×10^6 cells/ml TNE buffer.
2. Add EDTA (final concentration of 10 mmol/l), proteinase K (final concentration of 50 µg/ml; Merck, Darmstadt, FRG), and SDS (final concentration of 1% w/v) to the mixture.
3. Incubate the viscous mixture for 2 h. to overnight at 37 °C.
4. Extract the DNA with an equal volume of phenol extraction buffer (50% v/v high quality phenol; 49% v/v chloroform; 1% v/v isoamylalcohol).
5. Mix gently, until a homogenous solution is obtained.

NOTE: The solution containing the genomic DNA should be mixed gently to avoid fragmentation of the DNA.

6. Transfer the mixture to a polystyrene tube.
7. Centrifuge 5 min. at 2,000 × g.
8. Transfer the aqueous phase, containing the DNA, to an erlenmeyer.

NOTE: If admixture with interphase material occurs, the extraction procedure should be repeated once or twice.

9. Add 0.1 volume of 2 mol/l NaAc (pH 5.6) and 2 volumes of cold (-20°C) ethanol 96%.
10. Mix gently, until the DNA is precipitated.
11. Remove the precipitated DNA with a small glass rod.
12. Wash in 70% ethanol.
13. Dissolve the precipitated DNA in TE buffer (10 mmol/l Tris-HCl, pH 7.6; 1 mmol/l EDTA) overnight at 4°C.
14. Add DNase-free RNase (Boehringer Mannheim, Mannheim, FRG) to a final concentration of 20 µg/ml.
15. Incubate for at least 1 h. at 37°C.
16. Add proteinase K (final concentration of 25 µg/ml) and SDS (final concentration of 0.1% w/v).
17. Repeat steps 3 to 12.
18. Dissolve the DNA in 0.1 TE buffer (0.5-5 ml, depending on the amount of extracted DNA) at 4°C.

NOTE: Dissolving of the DNA usually takes 24-48 h.

19. Measure the optical density of the preparation with a spectrofotometer at 260 nm and 280 nm.

NOTE: The OD 260 is used to calculate the DNA concentration and the OD 280 to estimate the degree of protein contamination. High quality DNA should have an OD 260/280 ratio of at least 1.5.

Protocol 2. Restriction enzyme digestion of DNA

1. Pipet approximately 15-20 µg of genomic DNA (concentration 100-400 µg/ml) into a microcentrifuge tube.
2. Add 20 µl 10 × digestion buffer.
3. Add 8 µl spermidine (100 mmol/l; Sigma, St. Louis, MO).
4. Add 50 U (~3 U/µg DNA) of the appropriate restriction endonuclease.

NOTE: The type of digestion buffer (low, medium, or high ionic strength) depends on the type of restriction enzyme used and should be prepared according to manufacturer's guidelines. Spermidine is used to unfold the DNA.

5. Add aquadest to a final volume of 200 µl.
6. Mix the solution gently.
7. Briefly spin down the solution.
8. Incubate for 6 h. to overnight at 37°C.

NOTE: A control digestion to assess completeness of digestion is performed by adding 10 µl of the digestion mix to 1 µg of plasmid DNA in a separate microcentrifuge tube. This control digestion is incubated for 6 h. to overnight at 37 °C. The evaluation for the completeness of the control digestion is as follows:

9. Add one volume of TES buffer (10 mmol/l Tris-HCl, pH 7.6; 5 mmol/l EDTA; 0.1% w/v SDS) to the control digestion mixture.
10. Add 25 µl of phenol extraction buffer.
11. Vortex.
12. Centrifuge 3 min. at 15,000 x g.
13. Load the aqueous phase, supplemented with 5 µl of Orange G loading buffer (20% w/v Ficoll; 10 mmol/l Tris-HCl pH 7.6; 1 mg/ml Orange G) on a 0.7% agarose gel in TBE buffer (90 mmol/l Tris; 90 mmol/l boric acid; 2 mmol/l EDTA).

NOTE: If the banding pattern of the control digestion indicates that the digestion is complete, it is assumed that the digestion of the human DNA sample is also complete. If the control digestion is incomplete, an additional 50 units of restriction enzyme should be added to the genomic DNA mixture and incubated for a few hours to overnight. This second digestion of the genomic DNA should also be checked by a new control digestion.

If the control digestion is complete, the genomic DNA mixture can be prepared for electrophoresis.

14. Add 20 µl of 10 × ES buffer (50 mmol/l EDTA; 1% w/v SDS) to stop the reaction.
15. Add 200 µl phenol extraction buffer.
16. Centrifuge 3 min. 15,000 x g.
17. Transfer the aqueous phase to a clean tube.
18. Add 0.1 volume of 2 mol/l NaAc (pH 5.6) and two volumes of cold (–20°C) ethanol (96%).
19. Precipitate overnight at –20°C or 30 min. at -70°C.
20. Centrifuge 15 min. at 15,000 x g.
21. Wash the pellet in 70% ethanol.
22. Centrifuge 10 min. at 15,000 x g.
23. Air dry the pellet.
24. Dissolve the pellet in 21 µl H_2O.

NOTE: DNA concentrations can be measured to adjust the amount of DNA loaded in each lane.

25. Measure the optical density of 1 µl DNA sample at 260 nm to calculate the DNA concentration.
26. Adjust the DNA concentration in each sample, if needed.
27. Add 8 µl Orange G loading buffer to the DNA solution.

Protocol 3. Size separation of digested DNA by agarose gel electrophoresis

NOTE: The migration rate of a linear DNA fragment is influenced by the agarose concentration of the gel. By using gels of different concentrations, it is possible to resolve a wide size range of DNA molecules. For routine Southern blot analysis 0.7-0.8% agarose gels are used which optimally separate 1-15 kb fragments.

1. Dissolve agarose in TAE electrophoresis buffer (40 mmol/l Tris; 10 mmol/l EDTA; pH 8.2) supplemented with 0.5 µg/ml ethidium bromide.
2. Boil the solution in a microwave oven until it is clear.

NOTE: Several types of well-designed plexiglas electrophoresis equipment are commercially available. Many configurations and sizes of electrophoresis tanks, accompanying gel trays, and combs are available, dependent on personal preference.

3. Place the gel tray at a precisely horizontal position.
4. Place the combs and tray seals.
5. Seal the edges with a small volume of agarose solution using a pasteur pipette. Pour the agarose in the tray when it is approximately 50°C.
6. Carefully remove the combs and tray seals, after the gel is completely set.
7. Put the gel tray in the electrophoresis tank.

8. Add TAE buffer until it covers the gel with a fluid layer of 3-5 mm.
9. Load the samples into the slots.

NOTE: The two outer slots should be used for size markers. Markers with fragments in the range of 1 to 15 kb should be used.

10. Run the gel at 30-40 V (= 1-5 V/cm, measured the distance between the electrodes) overnight, until the tracking dye has migrated the appropriate distance through the gel.

NOTE: The resolution is better if the voltage is lower.

11. Examine the gel using a UV transilluminator (TM20; UVP, San Gabriel, CA).
12. Make a photograph of the gel.

NOTE: An UV ruler (Diversific Biotech, Newton Centre, MA) should be placed along side the gel for future estimation of the sizes of rearranged and germline bands.

Protocol 4. Transfer of DNA from gel to membrane

NOTE: Although there are different types of membranes we prefer nylon membranes, because they can be rehybridized several times, and because they bind nucleic acids irreversibly. The rate and the efficiency of the transfer of the DNA depends on the size of the DNA fragments; smaller fragments are transferred faster and more efficiently.

1. Soak the gel in 0.25 mol/l HCl for 10 min., while shaking gently.
2. Soak the gel twice in denaturing buffer (1.0 mol/l NaCl; 0.5 mol/l NaOH) for 15 min., while shaking gently.
3. Soak the gel twice in neutralization buffer (1.5 mol/l NaCl; 0.5 mol/l Tris, pH 7.0) for 15 min., while shaking gently.
4. Soak the gel in 10 × SSC transfer buffer (1.5 mol/l NaCl; 150 mmol/l sodium citrate, pH 7.0) for 15 min., while shaking gently.
5. Cut a piece of nylon membrane just large enough to cover the exposed surface of the gel, using gloves.
6. Soak the membrane briefly in aquadest and subsequently in 10 × SSC.

NOTE: Blotting can be performed by either vacuum transfer or capillary transfer. Vacuum blotting is less time consuming as the transfer is faster. There are several vacuum transfer devices commercially available (e.g. Vacu Gene XL; Pharmacia). If the gel is damaged the vacuum blotting procedure does not work and the more traditional capillary transfer should be applied. In the capillary blotting method the DNA is transferred via the mass flow of transfer buffer through the agarose gel to absorbent layers of paper on the top of the gel.

A. In case of vacuum transfer the following steps should be performed:

7a. Place the nylon membrane on a porous screen.
8a. Place the smooth side of the gel on top of the membrane.
9a. Pour 10 × SSC buffer on the gel, until the gel is covered with a fluid layer.
10a. Carry out blotting.

NOTE: A vacuum level of 30-35 mbar is sufficient for optimal blotting of a 0.7% agarose gel within 90 min.

B. In case of capillary transfer the following steps should be performed:

7b. Cut a large piece of GB004 blot paper (Schleicher and Schuell).
8b. Place the piece of GB004 blot paper on a glass plate, with the sides of the blot paper hanging into the 10 × SSC transfer buffer.
9b. Place the gel with the smooth side turned upwards on the wetted paper.
10b. Carefully place the nylon membrane on the gel.

NOTE: Avoid getting air bubbles by carefully rolling a glass pipet over the surface.

11b. Place 8 pieces of GB 002 blot paper on the membrane.
12b. Surround the gel with parafilm to prevent "short-circuiting" of fluid from GB004 blot paper to paper towels.
13b. Cut paper towels of a size similar to the gel.
14b. Place these paper towels on the GB 002 blot papers.
15b. Place a glass plate and a weight of 1 kg on the paper towels.
16b. Carry out blotting overnight.

NOTE: After blotting the DNA should be immobilized on the membrane. To our experience UV cross-linking generally results in a better fixation of the DNA as compared to baking at 80 °C.

17. Soak the nylon membrane in 10 × SSC to remove agarose, sticking to the filter, after the transfer is completed.
18. Air dry the membrane.
19. Expose the side with the DNA to 0.12 J UV in a 254 nm-UV crosslinker (Stratalinker, Stratagene, La Jolla, CA).

Protocol 5. Hybridization with ^{32}P labeled DNA probes

1. Prehybridize the membranes with 50 ml hybridization mixture (0.5 mol/l $NaHPO_4$, pH 7,2; 1% BSA Fraction V (Boehringer Mannheim), 1 mmol/l EDTA, 3% w/v SDS, 200 μg/ml sheared salmon sperm DNA) in a plastic box with a lid or in sealed plastic at 65 °C for 1 h., while shaking gently.

NOTE: Prehybridization is performed to reduce background signal by blocking non-specific binding sites.

2. Label the probe with ^{32}P.

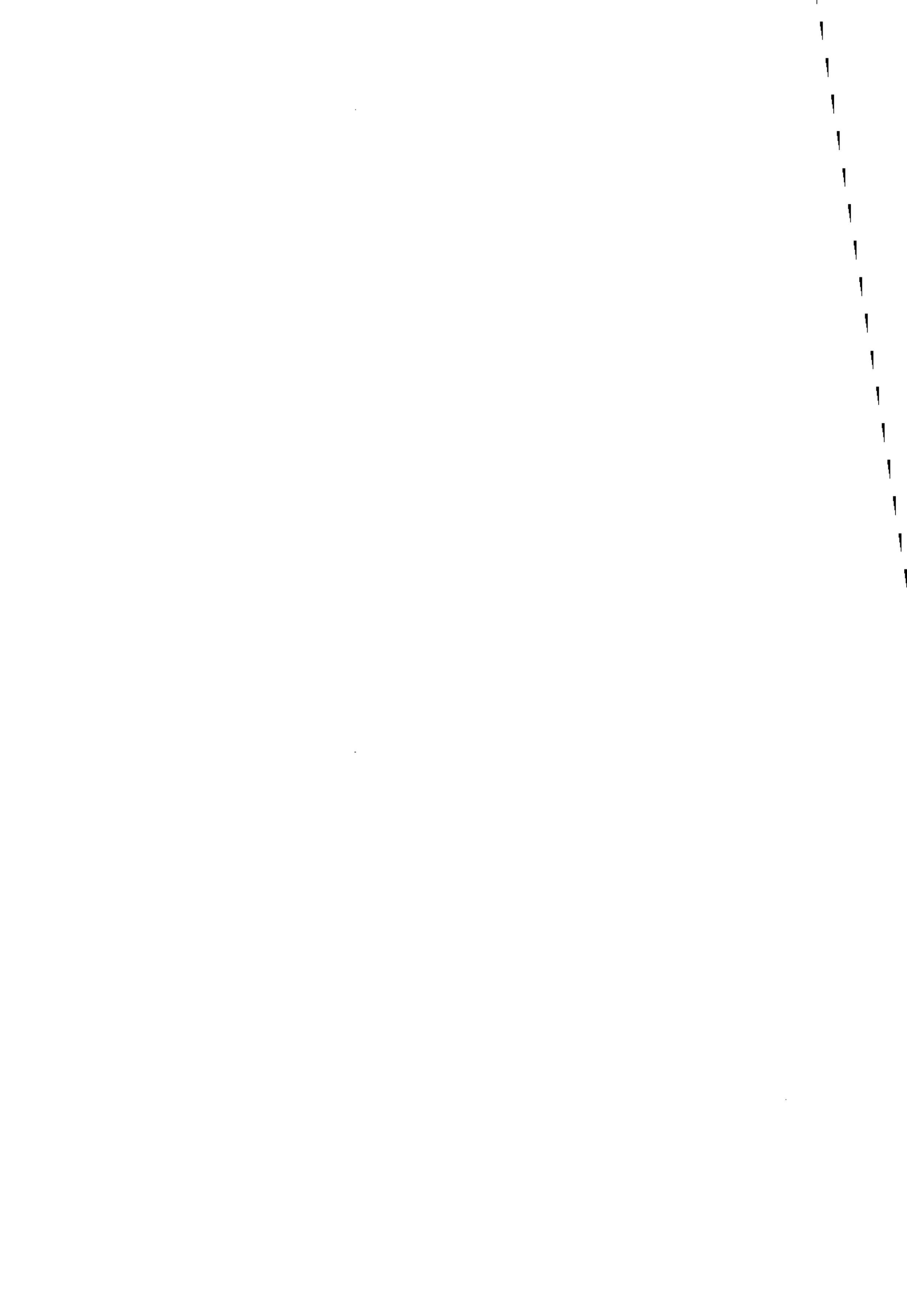
NOTE: The probes used for hybridization can be labeled by the nick-translation method or by random primer labeling (21, 22).

3. Denature the ^{32}P-labeled probe by boiling it for 3 min.
4. Cool the ^{32}P-labeled probe immediately on ice.
5. Add the ^{32}P-labeled probe to the hybridization mixture.
6. Hybridize the membrane overnight at 65°C, while shaking gently.
7. Wash the membrane once in wash buffer 1 (40 mmol/l $NaHPO_4$, pH 7.2; 2% w/v SDS; 1 mmol/l EDTA; 0.5% w/v BSA Fraction) for 5 min. at 65°C, followed by eight washes in wash buffer 2 (40 mmol/l $NaHPO_4$, pH 7.2; 1% w/v SDS; 1 mmol/l EDTA) for 3-5 min. at 65°C.
8. Rinse the membrane in 100 mmol/l $NaHPO_4$ (pH 7.2).
9. Briefly dry the membrane between paper layers.

NOTE: The membrane must not be dried too long, to avoid problems with probe removal prior to rehybridization.

10. Seal the membrane in a plastic bag.
11. Expose the membrane to an X-ray film in a cassette with intensifying screens at -80°C.

NOTE: Prior to rehybridization of the membrane, it should be washed in 50% deionized formamide and 6 × SSC for 30 min. at 65 °C, rinsed in 3 × SSC and soaked in 0.1 mmol/l $NaHPO_4$.



3

L'homme est né libre, et partout il est dans les fers (Rousseau)
De mens is vrij geboren, en toch moet hij ketenen dragen

CHAPTER 3

SOUTHERN BLOT DETECTION OF IMMUNOGLOBULIN LAMBDA LIGHT CHAIN GENE REARRANGEMENTS FOR CLONALITY STUDIES*

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SUMMARY

Southern blot analysis of immunoglobulin (Ig) genes has proven to be important for detection of clonal rearrangements in patients with lymphoproliferative diseases. To improve the detection of clonal Ig lambda (Igλ) gene rearrangements, we carefully determined the precise restriction map of the J-Cλ gene region, developed a suitable Cλ probe (IGLC3), and evaluated relevant restriction enzymes in combination with the IGLC3 probe. For the latter purpose, we selected 75 B-cell malignançies with proven expression of Igλ protein chains in order to be sure that each malignancy contained at least one clonally rearranged Igλ allele.

Our extensive Southern blot analyses with the IGLC3 probe in *Eco*RI and/or *Hin*dIII digests revealed that combined *Eco*RI/*Hin*dIII digestion detected Igλ gene rearrangements in 95% of the 75 patients and 94% of the 98 rearranged alleles. In contrast, *Hin*dIII and *Eco*RI single digests allowed detection of rearrangements in only 78% and 83% of the patients and 67% and 79% of rearranged alleles, respectively.

We conclude that the use of the IGLC3 probe in combined *Eco*RI/*Hin*dIII digests is superior to *Eco*RI and *Hin*dIII single digests. This probe/enzyme combination is informative for clonality studies in approximately 95% of patients.

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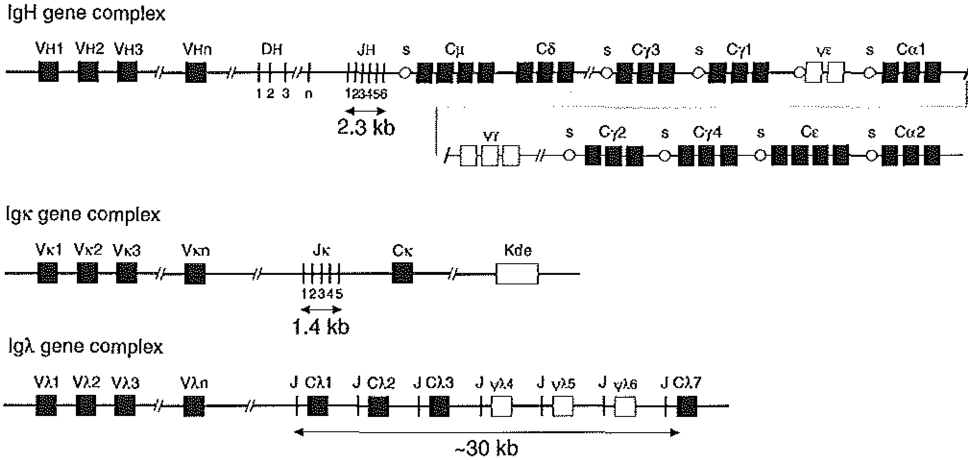

Figure 1. Schematic diagram of human Ig genes illustrating the location of the J gene segments in the different Ig gene complexes (1). The J gene segments in the IgH and Igκ gene complexes are clustered in relatively small regions of ~2.3 kb and ~1.4 kb, respectively. In contrast, the Jλ gene segments are scattered over a large region of ~30 kb.

INTRODUCTION

Lymphoid malignancies have a clonal origin and consequently malignant B-cells contain identically rearranged variable (V), diversity (D), and joining (J) gene segments of their immunoglobulin (Ig) genes (1). This concerns both the Ig heavy (IgH) genes and Ig light (IgL) genes (1-3). Therefore Ig genes can be used as target for clonality studies in lymphoproliferative diseases of B-cell origin (4).

Southern blot analysis of IgH and Igκ genes for detection of clonal rearrangements of J gene segments is easy, because all J gene segments cluster in relatively small regions of ~2.3 kb and ~1.4 kb, respectively (Figure 1)(1). However, Southern blot analysis of Igλ gene rearrangements is difficult, because the seven Jλ gene segments are scattered over a large region of ~30 kb (Figure 1)(1,5-10). This would imply that multiple Jλ gene probes are needed to detect all Jλ gene rearrangements, which is laborious and time consuming. Therefore generally a single constant (C) exon probe is used, which recognizes all Cλ exons due to high homology of ~85% (1,5-10). This approach is hampered by four limitations. Firstly, for detection of J gene rearrangements with a Cλ probe only restriction enzymes without cut sites in the seven J-Cλ introns can be used, such as *Eco*RI and *Hin*dIII (1,5,6,8). Secondly, the Cλ probe does not only recognize Cλ exons of the classical Igλ locus (Figure 1) (1,5-10), but also cross-hybridizes to Cλ exons of the surrogate λ-like gene complex, 14.1, 16.1, 16.2, and 18.2 (11-15). Thirdly, rearranged bands might comigrate with one of the multiple Cλ germline bands, especially in case of large rearranged and germline fragments (1); finally, a genetic amplification polymorphism in the Cλ2-Cλ3 gene region causes extra bands, which make the interpretation of the Southern blots even more complicated (Figure 2)(1,16,17).

Igλ gene rearrangements occur in at least one third of all B-cell malignancies (4,18).

Therefore we wished to improve the Southern blot detection of clonal Igλ gene rearrangements. We carefully determined the Igλ gene restriction map, designed a suitable Cλ probe, and evaluated several probe/enzyme combinations to determine the optimal combination for routine Southern blot detection of clonal Igλ gene rearrangements. For this purpose we selected a large series of 75 B-cell malignancies with expression of Igλ protein chains to be sure that each malignancy contained at least one clonally rearranged Igλ allele.

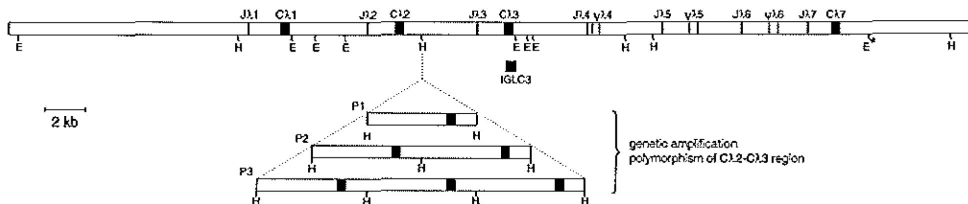

Figure 2. Organization of the J and C gene segments of the human Igλ gene complex, including the genetic amplification polymorphism of the J-Cλ2/J-Cλ3 gene region (1,5,6,8,16). The location of the relevant *Eco*RI (E) and *Hin*dIII (H) restriction sites are indicated. The asterisk indicates the resistant *Eco*RI site downstream of the Cλ7 exon. The solid boxes represent functional Cλ exons and dotted boxes are non-functional (pseudo; ψ) Cλ exons. The location of the IGLC3 probe is indicated as a solid bar; this probe recognizes all Cλ exons of the classical Igλ gene complex and the surrogate λ-like gene complex.

MATERIALS AND METHODS

Cell samples

Mononuclear cells (MNC) were obtained from peripheral blood (PB) and bone marrow (BM) samples by Ficoll-Paque (density: 1,077 g/l; Pharmacia, Uppsala, Sweden) centrifugation from a large series of ~250 patients with a mature Ig^+ B-cell malignancy, including B-cell chronic lymphocytic leukemia (B-CLL), B-cell prolymphocytic leukemia (B-PLL), hairy cell leukemia (HCL), B-cell non-Hodgkin lymphoma (B-NHL), and multiple myeloma. The MNC were used for detailed immunophenotyping, including detection of surface membrane and intracellular IgH and IgL expression (19,20). Remaining cells were stored in liquid nitrogen. We selected MNC from 75 patients with an $Ig\lambda^+$ B-cell malignancy, i.e. 56 chronic B-cell leukemias (tumor load >70%), 12 B-NHL (tumor load >70%), and 7 multiple myelomas (tumor load >25%).

Control cell samples (granulocytes, cell lines, and MNC) without clonal Igλ gene rearrangements were used for determining the occurrence of restriction fragment length polymorphisms (RFLP).

Southern blot analysis

DNA was isolated as previously described (1,21). Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-fractionated in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleichler and Schuell, Dassel, Germany) (1). The membranes were hybridized with the ^{32}P-random oligonucleotide labeled probe.

Restriction map

The major part of our restriction map could be based on information by Blomberg et al. (6,10) and sequence data by Vasicek et al., i.e. starting from 2429 bp upstream of the Jλ1 gene segment to 2100 bp downstream of the Cλ7 gene segment (8). Additional information was obtained by our extensive Southern blot analyses and careful calculations of germline restriction fragments, including detailed evaluation of discrepancies in restriction maps from the literature.

Construction of IGLC3 probe

The IGLC3 probe was obtained by cloning the purified polymerase chain reaction (PCR) amplification product of granulocyte DNA from a healthy volunteer. The oligonucleotide primers were synthesized according to published sequences of the Cλ3 region (8) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA,USA) with the solid-phase phosphodiester method and used without further purification. The sequences (with aspecific tails containing *Hin*dIII and *Eco*RI restriction sites for cloning) were for the upstream primer: 5′ TCCTCTGAGAAGCTTCAAGCCAAC 3′ and for the downstream primer: 5′ ACTGGGTGCAGAATTCCCTCCAC 3′. PCR was essentially performed as previously described (21). An 1.0 μg sample of granulocyte DNA, 12.5 pmol of the upstream and downstream primers, and one unit of *AmpliTaq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT,USA) were used. The PCR products were size-fractionated 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 digested with *Hin*dIII and *Eco*RI and cloned, using pUC19 as cloning vector (21,22). The probe was sequenced from both sides (at least 100 bp) to confirm its position in the Cλ3 region and to exclude cloning artifacts. Sequencing was performed with the T7-sequencing kit (Pharmacia) following the manufacturer's instructions using ^{35}S radiolabeling and run in a denaturing 8% polyacrylamide sequence gel (21). The IGLC3 probe is a general Cλ probe of 490 bp which recognizes all Cλ exons in the classical Igλ gene locus and Cλ exons of the surrogate λ-like genes.

RESULTS

Completion of the restriction map

The major part of the restriction map of the J-Cλ locus was obtained from the literature (1,5,6,8,10). For this study we focussed on *Eco*RI and *Hin*dIII restriction sites, because these enzymes do not cut in the J-Cλ introns and therefore can be used for detection of Jλ gene rearrangements in combination with the IGLC3 probe (Figure 2) (1). In *Eco*RI digests the J-Cλ1 gene region and the J-Cλ2/J-Cλ3 gene region reside on two separated restriction fragments, while the J-Cλ4/ J-Cλ5/ J-Cλ6/ J-Cλ7 gene segments reside on a single restriction fragment (Table 1 and Figure 2). In *Hin*dIII digests the J-Cλ1/J-Cλ2 gene region and the J-Cλ3/J-Cλ4 gene region are located on two separate fragments, while the J-Cλ5/ J-Cλ6/ J-Cλ7 gene segments are on a single fragment (Table 1 and Figure 2). In combined *Eco*RI/*Hin*dIII digests J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ4 gene segments reside on separate restriction fragments, while the J-Cλ5/ J-Cλ6/ J-Cλ7 gene region resides on one restriction fragment (Table 1 and Figure 2).

Our Southern blot data were not fully in line with the sequence data by Vasicek et al. with respect to the supposed *Eco*RI restriction site located 1.76 kb downstream of Cλ3 exon (8). In *Eco*RI/*Hin*dIII digests this would result in an J-Cλ4 fragment of 3.8 kb. However, we observed a fragment of 4.5 kb. To prove the absence of this *Eco*RI restriction site, we performed PCR from 6 bp to 1974 bp downstream of Cλ3 exon which covers several relevant *Eco*RI restriction sites (Figure 2). The PCR products were digested with *Eco*RI and run on 8% polyacrylamide gels and 2% agarose gels. We found that the predicted *Eco*RI site 1.76 kb downstream of Cλ3 exon does not exist, which is in line with our Southern blot data.

Furthermore, we investigated the *Eco*RI site downstream of the Cλ7 exon by use a special Jλ7 probe (IGLJ7 probe) (Tümkaya et al., unpublished results). This restriction

TABLE 1. IGLC3 DNA probe and restriction enzymes for detection of Igλ gene rearrangements.

Appropriate restriction enzymes for digestion of genomic DNA	Size of germline restriction fragments in kb	Allelic frequencies of polymorphisms	
*Hin*dIII	9.0 (λ1 and λ2); 10.1 (λ3 and ψλ4); 14.6 (ψλ5, ψλ6 and λ7)	0% (0/126)	except 5.4 kb in case of amplification polymorphism of J-Cλ2/J-Cλ3
*Eco*RI	13.5 (λ1); 8.4 (λ2 and λ3); 15.8 (ψλ4, ψλ5, ψλ6 and λ7)	0% (0/56)	except 13.7 kb; 19.1 kb; 24.5 kb in case of amplification polymorphism of J-Cλ2/J-Cλ3
*Eco*RI-*Hin*dIII	2.6 (λ1); 3.8 (λ2); 4.6 (λ3); 4.7 (ψλ4); 10.5 (ψλ5, ψλ6 and λ7)	0% (0/112)	except 5.4 kb in case of amplification polymorphism of J-Cλ2/J-Cλ3

* We assumed that the combined results of the *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII digests allowed the detection of (virtually) all Igλ gene rearrangements, since we identified at least one rearranged Igλ allele in all 75 patients. And two (n=21) or three (n=1) rearranged Igλ alleles in 29% (of patients).

site appeared to be frequently resistant to digestion,resulting in an extra band of ~14.5 kb in *Eco*RI/*Hin*dIII digests and ~28 kb in *Eco*RI digests (Figures 2 and 3).

Genetic polymorphisms

It has been reported that due to a genetic amplification polymorphism, part of the Cλ2-Cλ3 gene region might be amplified one to three times (1,16,17). In *Eco*RI digests each amplification gives an extension of 5.4 kb of the Cλ2-Cλ3 restriction fragment, while the amplified fragments appear as separate 5.4 kb restriction fragments in *Hin*dIII digests (Figures 2 and 3) (1,16,17). In combined *Eco*RI/*Hin*dIII digests this 5.4 kb fragment comigrates with a germline Cλ band of the surrogate λ-like locus (Table 1 and Figure 3). Analysis of germline DNA samples with the IGLC3 probe in *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII digests did not reveal the occurrence of other polymorphisms, such as RFLP (Table 1).

Southern blot analysis of patient samples

To evaluate the most optimal combinations of the IGLC3 probe with *Eco*RI and/or *Hin*dIII enzymes, we tested these combinations in 75 patients with an Igλ$^+$ B-cell malignancy (Table 2). We assumed that the use of the IGLC3 probe in combination with three digests (*Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII) would allow us to detect all or virtually all Igλ gene rearrangements in the 75 Igλ$^+$ B-cell malignancies. Indeed we detected at least one clonally rearranged Igλ allele in each patient with a total of 98 rearranged alleles. However, the detectability of the rearrangements was highly dependant on the digest: the *Hin*dIII, *Eco*RI, and *Eco*RI/*Hin*dIII digests detected clonal rearrangements in 76%, 83%, and 95% of patients, respectively, which represented 67%, 79%, and 94% of the rearranged alleles, respectively (Table 2).

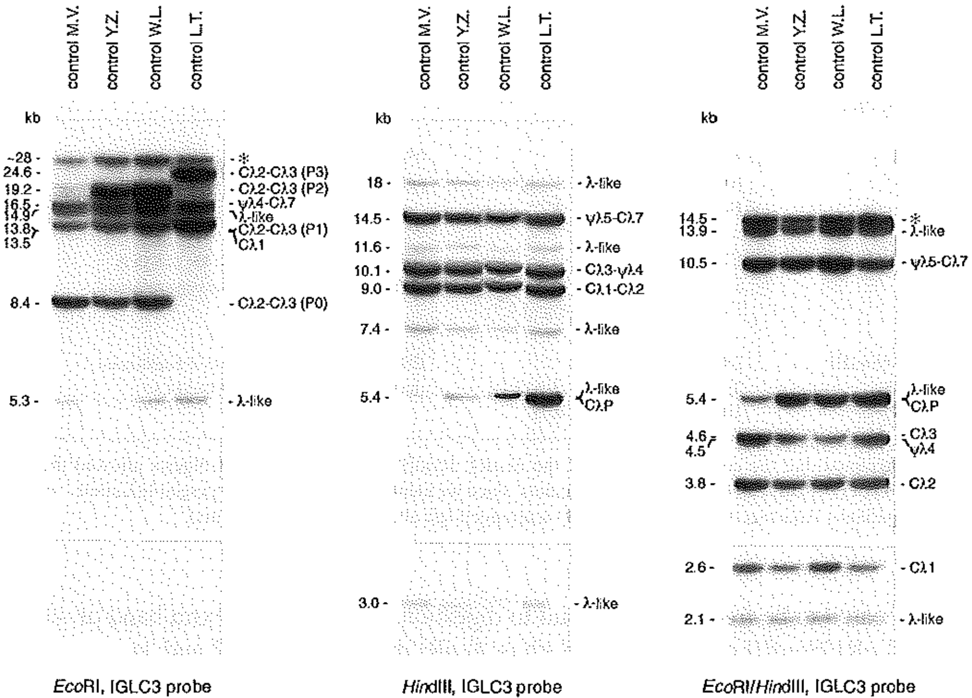

Figure 3. Southern blot analysis of the Igλ genes. DNA samples from different controls were digested with *Eco*RI and/or *Hin*dIII, size-fractionated, blotted onto a nylon membrane, and hybridized with the ^{32}P-labeled IGLC3 probe. This figure illustrates the complex banding patterns of *Eco*RI, *Hin*dIII, and combined *Eco*RI/*Hin*dIII digests. The various germline fragments of the classical Igλ locus as well as the faint germline bands of the surrogate λ-like gene complex are indicated. In the *Eco*RI and *Hin*dIII single digests the germline fragments are larger than in the combined *Eco*RI/*Hin*dIII digests. Also the banding pattern of the genetic amplification polymorphism of the Cλ2-Cλ3 region is illustrated. The first lane of each digest contains control DNA without amplification polymorphism (P0/P0); lane 2 and lane 3 have amplification polymorphisms on one allele (P0/P2); lane 4 has amplification polymorphisms on both alleles (P1/P3). Each amplification gives an extension of the Cλ2-Cλ3 restriction fragment with 5.4 kb in *Eco*RI digests, but in *Hin*dIII and *Eco*RI/*Hin*dIII digests the polymorphic amplification results in a single 5.4 kb band (Table 1). The asterisk indicates the resistant *Eco*RI site downstream of the Cλ7 exon (Figure 2).

DISCUSSION

Although little attention has been paid to the Igλ gene complex, it is an important target for clonality studies in at least one third of B-lineage malignancies (4). Therefore we wished to improve Southern blot analysis of Igλ genes for diagnostic clonality studies. For this purpose we developed the Cλ probe, IGLC3, and we selected suitable restriction enzymes for detection of Jλ rearrangements, i.e. *Eco*RI, *Hin*dIII, and combined *Eco*RI/*Hin*dIII digests. The *Eco*RI and *Hin*dIII single digests appeared to cause several problems. Firstly, the germline fragments are relatively large, causing co-migration of germline and rearranged bands due to insufficient separation (1). Secondly, many cross-hybridizing fragments of the surrogate λ-like genes have comparable sizes as the germline fragments

of the classical Igλ locus; this further hampers the identification of rearranged bands (11,14,15). Thirdly, generally two or more J-Cλ gene regions reside on the same restriction fragment in *Eco*RI and *Hin*dIII digests (Figure 2). For optimal analysis it would be better that the functional J-Cλ gene regions (i.e. J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 regions) are located on separate restriction fragments.

In addition to *Eco*RI and *Hin*dIII single digests, we tested combined *Eco*RI/*Hin*dIII digests in order to solve many of the above problems: the germline fragments are smaller, resulting in better separation; most of the cross-hybridizing fragments are outside the normal range of germline fragments; and the individual functional J-Cλ gene regions are located on separate fragments, thereby facilitating the interpretation of the banding patterns (Figure 3).

To determine the optimal probe/enzyme combinations, we analysed 75 proven Igλ+ clonal B-cell malignancies with the IGLC3 probe in *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII digests (Figure 4). We assumed that the combination of *Eco*RI, *Hin*dIII, and *Eco*RI/

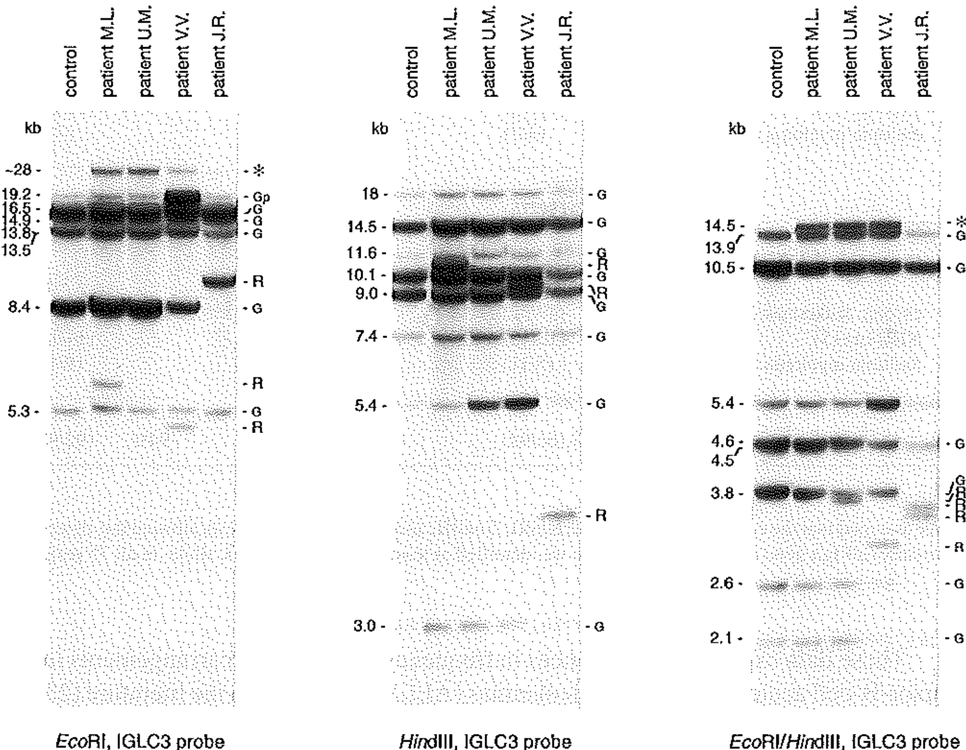

Figure 4. Southern blot analysis of Igλ genes in Igλ+ B-cell malignancies. The DNA samples were digested with *Eco*RI, *Hin*dIII, or *Eco*RI/*Hin*dIII, size-fractionated, blotted onto a nylon membrane, and hybridized with the ^{32}P-labeled IGLC3 probe. Lane 1 contains control DNA and shows the germline (G) bands. Lane 2 contains DNA with a clonal Igλ gene rearrangement, which was detectable with *Eco*RI and *Hin*dIII digests, but exceptionally not with the combined *Eco*RI/*Hin*dIII digest. The rearrangement banding patterns in lanes 3, 4, and 5 illustrate that optimal detection of Igλ gene rearrangements is possible with combined *Eco*RI/*Hin*dIII digests (Table 2). The asterisk indicates the resistant *Eco*RI site downstream of the Cλ7 exon (Figure 2).

*Hin*dIII digests would allow us to detect all Igλ gene rearrangements in our series of 75 patients. Indeed the combined results revealed that all 75 patients contained clonally rearranged Igλ genes: 53 patients had monoallelic rearrangements, 21 patients had biallelic rearrangements, and one patient showed three rearranged bands. The latter might be due to a numerical or structural aberration of chromosome 22. The extensive Southern blot study demonstrated that combined *Eco*RI/*Hin*dIII digests are most effective for detection of Igλ gene rearrangements, because the *Hin*dIII, *Eco*RI, and *Eco*RI/*Hin*dIII digests detected at least one rearranged allele in 76%, 83%, and 95% of patients, respectively (Table 2). Also evaluation at the level of Igλ alleles revealed that *Eco*RI/*Hin*dIII digests are superior, because the *Hin*dIII, *Eco*RI, and *Eco*RI/*Hin*dIII digests detected 67%, 79%, and 94% of rearranged alleles, respectively (Table 2).

TABLE 2. Detection of rearrangements in *Eco*RI and *Hin*dIII digests with the IGLC3 probe*.

	*Hin*dIII	*Eco*RI	*Eco*RI/*Hin*dIII
Igλ+ B-cell malignancies			
chronic leukemias (n=56)	(42/56)	(45/56)	(53/56)
NHL (n=12)	(11/12)	(11/12)	(11/12)
multiple myeloma (n=7)	(4/7)	(6/7)	(7/7)
	76% (57/75)	83% (62/75)	95% (71/75)
Rearranged Igλ alleles			
chronic leukemias (n=72)	(49/72)	(54/72)	(69/72)
NHL (n=15)	(12/15)	(14/15)	(14/15)
multiple myeloma (n=11)	(5/11)	(9/11)	(9/11)
	67% (66/98)	79% (77/98)	94% (92/98)

* We assumed that the combined results of the *Eco*RI, *Hin*dIII, and *Eco*RI/*Hin*dIII digests allowed the detection of (virtually) all Igλ gene rearrangements, since we identified at least one rearranged Igλ allele in all 75 patients.

These data explain the differences in frequency of Igλ gene rearrangements in precursor-B-cell acute lymphoblastic leukemias (ALL), as reported in the literature (23-26). We used a Cλ probe in *Eco*RI/*Hin*dIII digests and found Igλ gene rearrangements in 20-25% of precursor-B-ALL cases (18). However, in virtually all other studies a Cλ probe was used in *Eco*RI digests, resulting in essentially lower frequencies (~5%) of Igλ gene rearrangements in precursor-B-ALL (23-26).

We conclude that Southern blot detection of Igλ gene rearrangements for diagnostic clonality studies needs the use of a Cλ DNA probe (e.g. the IGLC3 probe) in combination with *Eco*RI/*Hin*dIII digests, because this combination allows detection of clonally rearranged Igλ genes in 95% of Igλ+ B-cell malignancies.

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4

Where's life, there is dynamism

CHAPTER 4

IDENTIFICATION OF IMMUNOGLOBULIN LAMBDA ISOTYPE GENE REARRANGEMENTS BY SOUTHERN BLOT ANALYSIS*

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SUMMARY

The human immunoglobulin lambda (Igλ) gene locus contains seven homologous Cλ exons which are organized in a tandem array, each of which is preceded by a single Jλ gene segment. The J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 are functional gene regions and encode for the four Igλ isotypes, whereas the J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) Igλ gene regions.

Recently, we demonstrated that Southern blot analysis with the IGLC3 probe in combined *Eco*RI/*Hin*dIII digests allows detection of approximately 95% of all clonal Igλ gene rearrangements in B-cell malignancies. Although this single probe/enzyme combination is quite effective in detecting Igλ gene rearrangements, it should be noted that it results in a complex pattern of multiple germline bands of different density, which needs experience for correct interpretation. To further improve the reliable detection and identification of clonal Igλ gene rearrangements, we developed a new set of seven "isotype-specific" DNA probes: the IGLC1D probe for the J-Cλ1 gene region, the IGLC2D probe for the J-Cλ2 gene region, the IGLJ2 probe for the highly homologous J-Cλ2 and J-Cλ3 gene regions, and the IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes for the last four J-Cλ gene regions, respectively. In combination with optimally chosen digests (i.e. *Hin*dIII, *Bgl*II, *Bam*HI, and/or *Eco*RI) the seven probes indeed allow easy detection and identification of all rearrangements in the seven J-Cλ gene regions. The applicability of the probe/enzyme combinations was confirmed upon analysis of clonal "Igλ isotype" gene rearrangements in 40 B-lineage malignancies.

INTRODUCTION

The classical human immunoglobulin lambda (Igλ) locus contains seven Cλ gene segments, each preceded by a J gene segment, which are spread over a total area of approximately 30 kb (1-3) . The J-Cλ1, J-Cλ2, J-Cλ3 and J-Cλ7 regions are functional and code for the four distinct Igλ isotypes, whereas J-Cλ4, J-Cλ5 and J-Cλ6 regions are not functional due to deletions and/or insertions in the Cλ gene segments (4-6). The seven J-Cλ regions are homologous; this especially concerns the J-Cλ2 and J-Cλ3 gene regions with a homology of 98% (1,2,4-6).

Because at least one third of all B-cell malignancies have rearranged their Igλ genes, they can be useful for clonality studies (7). Therefore, we recently evaluated the use of a Cλ probe (IGLC3) for Southern blot detection of clonal Igλ gene rearrangements (Figure 1) (8). The IGLC3 probe hybridizes to all Cλ gene segments of the classical Igλ locus as well as the Cλ gene segments of the surrogate Igλ locus (1-6,9,10). We demonstrated that the IGLC3 probe in combined *Eco*RI/*Hin*dIII digests detects approximately 95% of all Igλ gene rearrangements in B-cell malignancies, indicating that this single probe/enzyme combination is quite effective in detecting Igλ gene rearrangements (8). The remaining 5% of Igλ gene rearrangements are probably missed due to comigration of the rearranged bands with one of the multiple germline bands, which represent the Cλ gene segments of the classical and surrogate Igλ loci. Due to differences in homology between the various Cλ gene segments, hybridization with the IGLC3 probe leads to germline bands of different density. This results in complex banding patterns, which need experience for correct interpretation (8).

We wished to further improve the detection of Igλ gene rearrangements and to identify the J-Cλ regions, involved in the rearrangements. Therefore we developed a set of seven "Igλ-isotype-specific" DNA probes, which allow reliable detection of Igλ

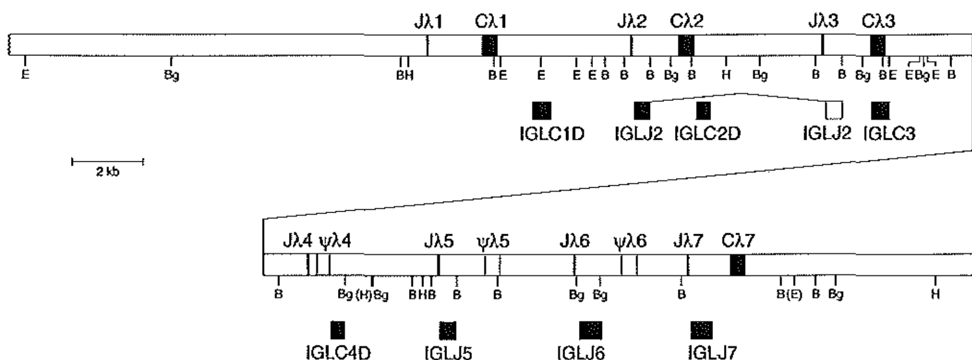

Figure 1. Organization of the J and C gene segments of the human Igλ gene complex. The location of the relevant *Hin*dIII (H), *Bgl*II (Bg), *Bam*HI (B), and *EcoRI* (E) restriction sites is indicated (see reference 5). The partially resistant *Hin*dIII and *Eco*RI restriction sites downstream of the ψCλ4 and Cλ7 exons, respectively, are in parentheses. The solid boxes represent the functional Cλ exons and the dotted boxes represent the non-functional (pseudo; ψ) Cλ exons. The location of the probes is indicated by solid bars. These probes are specific for the corresponding J-Cλ gene segments, except for the IGLJ2 probe, which recognizes both J-Cλ2 and J-Cλ3 gene regions due to a high homology of 98% (recognition site in the J-Cλ3 region is indicated with an open bar).

gene rearrangements to support investigation of differential Igλ isotype usage in B-cell malignancies. We carefully determined optimal restriction enzyme digests for each probe and evaluated the occurrence of restriction fragment length polymorphisms (RFLP) for these probe/enzyme combinations.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) samples of 73 healthy adult volunteers were used for isolation of granulocytes from the cell pellet after Ficoll-Paque centrifugation (density: 1,077 g/l; Pharmacia, Uppsala, Sweden). The granulocyte cell samples were frozen and stored in liquid nitrogen to be used for DNA extraction for RFLP studies. As positive controls for rearranged Igλ genes, we selected 40 B-lineage malignancies: 20 precursor-B-acute lymphoblastic leukemias (precursor-B-ALL), 10 $Ig\lambda^+$ chronic lymphocytic leukemias (B-CLL), and 10 $Ig\lambda^+$ multiple myelomas (8,11).

Southern blot analysis

DNA was isolated as previously described (3,12). Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-fractioned in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Scheichler and Schuell, Dassel, Germany) (3). The membranes were hybridized with ^{32}P-random oligonucleotide labeled probes (3).

Restriction map and polymorphisms

The whole sequence of the J-Cλ locus, starting from 2429 bp upstream of the Jλ1 gene segment to 2100 bp downstream of Cλ7 gene segment, was determined by Vasicek and Leder (5). Granulocyte DNA samples were used for digestion with the restriction enzymes *Hin*dIII, *Bgl*II, *Bam*HI, and/or *Eco*RI (Pharmacia) in order to confirm the precise position of the restriction sites within the J-Cλ locus and to assess the occurrence of RFLP in combination with the seven Igλ DNA probes.

Construction of Igλ DNA probes

We constructed seven new DNA probes for optimal detection and identification of Igλ gene rearrangements. The probes were obtained by cloning the purified polymerase chain reaction (PCR) amplification products of granulocyte DNA. The oligonucleotide primers contained aspecific tails with *Eco*RI, *Hin*dIII, *Bam*HI, or *Bgl*II restriction sites for cloning (Table 1). All oligonucleotide primers were synthesized according to published sequences of the J-Cλ regions (5) and EMBL databank (accession number X51755) on a 392 DNA synthesizer (Applied Biosystems, Forster City, CA, USA) with the solid-phase phosphodiester method and used without further purification. PCR was essentially performed as previously described (12,13). 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 size-fractioned 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 digested with the appropriate restriction enzymes and cloned, using pUC19 as cloning vector (12,13). All seven probes were sequenced from both sides (at least 100 bp) to confirm their position in the J-Cλ region and to exclude cloning artifacts. Sequencing was essentially performed as described elsewhere (12,13). All sequence reactions were performed with the T7-sequencing kit (Pharmacia) following the manufactor's instructions using ^{35}S radiolabeling, and run in normal denaturing 8% polyacrylamide sequence gels.

TABLE 1. Oligonucleotide primers used for construction of the human Igλ gene DNA probes.

Probe code[a]	Size	Cloning sites	Primer code	Relative position[b]	Sequence[c] 5′ → 3′
IGLC1D	534 bp	*Bam*HI/*Hin*dIII	Cλ1dp5′	3058	TTGGGGATCcGGCTCAAAGTTAACA
			Cλ1dp3′	3547	AgtGaaGcTtCGAGAGTACCCAGGCACTGAGG
IGLJ2	449 bp	*Hin*dIII/*Bam*HII	Jλ2-p5′	129	TTCTGaAgCTTGTCTCAACTTGTGGTCAGC
			Jλ2-p3′	533	GTCTGGATCCTGGCTCTGGGTC
IGLC2D	390 bp	*Hin*dIII/*Eco*RI	Cλ2d-p5′	1898	TCTaAgcTTGTGGTGGAAAGAACCCTGAACC
			Cλ2d-p3′	2245	TCATGaATTCTCCTGACACAGAGAGC
IGLC4D	378 bp	*Hin*dIII/*Bgl*II	Cλ4d-p5′	670	TgtcaagCTTATCTCATATTTAGTTTGCAA
			Cλ4d-p3′	1022	GTTGAGATCTCAGCCACGTGCTG
IGLJ5	475 bp	*Hin*dIII/*Bam*HI	Jλ5-p5′	77	gtgtaagCTTCCCTGGTCTCCCCAAGGTA
			Jλ5-p3′	520	AGCCTGGATCCAGAGTCCCACA
IGLJ6	647 bp	*Eco*RI/*Hin*dIII	Jλ6-p5′	176	CTGGCCCCgAATTCCTCCAGCC
			Jλ6-p3′	786	TGtgaaGCTTGCATGTGAGGTATATTTTCT
IGLJ7	535 bp	*Hin*dIII/*Eco*RI	Jλ7-p5′	139	CTAAGGTCTAAGCtTGTCTGGATG
			Jλ7-p3′	601	CACCCgAatTCCCTGCAGAGACCCCTCTTG

a. The position of the DNA probes is indicated in Figure 1.
b. The position of the 3′side of the oligonucleotide primers is indicated relative to the 3′side of the recombination signal sequence of the involved Jλ gene segment.
c. The lower case characters represent aspecific nucleotides, which generate restriction sites (underlined). Sequence information used to design the oligonucleotide primers was derived from Vasicek and Leder (5) and from EMBL databank accession number X51755.

RESULTS AND DISCUSSION

Although the structure of the human IgH and Igκ genes in B-cell malignancies has been studied extensively during the last decade, less attention has been paid to rearrangements in the human Igλ light chain locus. To allow optimal detection and identification of Igλ gene rearrangements, we developed a new set of seven "Igλ-isotype-specific" DNA probes. These probes were designed according to the following criteria: firstly, no cross-hybridization to other DNA fragments should occur; secondly, the size of the probes should be between 500 bp and 1 kb; thirdly, if possible, the probes should be positioned immediately downstream of the corresponding Jλ gene segments; finally, for each probe at least two appropriate restriction enzyme digests should be selected, which result in small restriction fragments (preferably between 2 and 10 kb) to overcome the problem of insufficient separation or comigration of rearranged and germline bands (3,14).

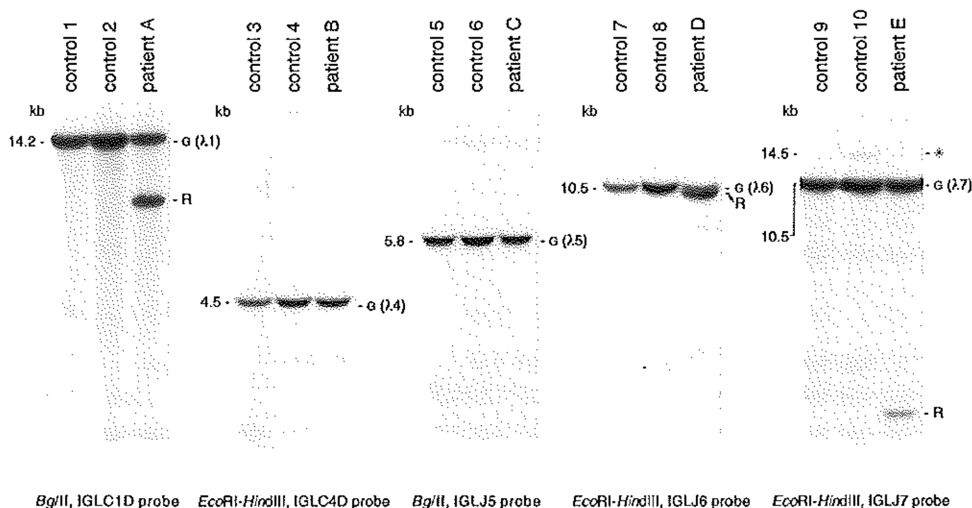

Figure 2. Southern blot analysis of the Igλ genes with IGLC1D, IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes. DNA samples from different controls and B-lineage malignancies with Igλ gene rearrangements were digested with *Bgl*II or *Eco*RI/*Hin*dIII, size fractionated, blotted onto a nylon membrane, and hybridized with the ^{32}P-labeled DNA probes. The first two lanes of each DNA filter contain control DNA samples, resulting in germline (G) bands. Each third lane contains DNA from patients with different Igλ-isotype gene rearrangements (8,9). The DNA filters show the occurrence of rearrangements in the J-Cλ1, J-Cλ6, and J-Cλ7 regions, but no rearrangements were found in the J-Cλ4 and J-Cλ5 gene regions in our analysis of 40 B lineage malignancies. The asterisk in the IGLJ7 probe panel indicates the 14.5 kb band, as a result of the partially resistant *Eco*RI site downstream of the Cλ7 gene segment (8).

So far, a few "Igλ-isotype-specific" probes have been published (e.g. pJλ2 and pJλ6) (4,15), but they are not suitable for rearrangement studies, because a large part of these probes recognize sequences upstream of the corresponding Jλ gene segments, which will be deleted upon rearrangement (3,14). According to the above criteria for probe design, we tried to position our probes immediately downstream of the corresponding Jλ gene segments in the J-Cλ intron. This appeared to be possible for the J-Cλ2, J-Cλ3, J-Cλ5, J-Cλ6, and J-Cλ7 regions, resulting in the IGLJ2, IGLJ5, IGLJ6, and IGLJ7 probes (Figure 1). Because of the high homology between the J-Cλ2 and J-Cλ3 gene regions, the IGLJ2 probe recognizes both regions. Therefore Jλ2 and Jλ3 rearrangements can only be identified with the IGLJ2 probe by combined evaluation of the rearranged bands and estimation of the density of the remaining germline bands. We found this to be difficult or impossible, when the B-cell tumor load is less than 70 to 80%. To facilitate discrimination between Jλ2 and Jλ3 rearrangements, we designed the λ2-specific IGLC2D probe, which exclusively recognizes sequences downstream of the Cλ2 exon (Figure 1); this region is not homologous to the downstream Cλ3 region (5). For the J-Cλ1 and J-Cλ4 regions it was not possible to fulfill our criteria for probe design. Initially, we developed three different probes in the J-Cλ1 intron, but all of them appeared to cross-hybridize with other genomic DNA fragments, probably located in the surrogate Igλ-like locus (9,10).

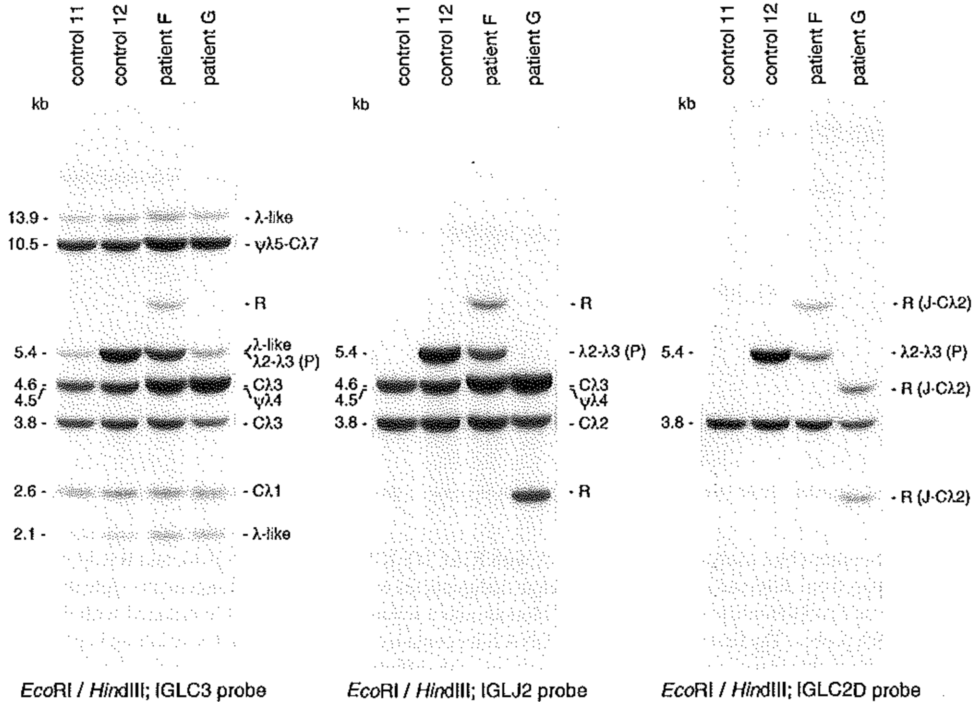

Figure 3. Southern blot analysis of Igλ genes in controls and B-lineage malignancies by successive hybridization of an *Eco*RI/*Hin*dIII filter with the IGLC3, IGLJ2, and IGLC2D probes. The rearrangements detected with the IGLJ2 probe were all detectable with the IGLC2D probe, indicating that they concerned Jλ2 rearrangements.

To overcom this problem, we successfully positioned the λ1-specific probe downstream of the Cλ1 exon (IGLC1D probe in Figure 1). In case of the J-Cλ4 gene region, the intervening sequence between Jλ4 and Cλ4 gene segments is too short to develop an appropriate probe according to our definitions (Figure 1). Therefore we positioned the λ4-specific probe just downstream of the Cλ4 exon (IGLC4D probe in Figure 1).

The *Bgl*II, *Hin*dIII, *Bam*HI, and *Eco*RI restriction sites in the J-Cλ gene locus were obtained from sequence data in the literature (5). Furthermore, we determined the position of the *Bgl*II and *Eco*RI restriction sites upstream of the Jλ1 gene segment and the *Hin*dIII and *Bgl*II restriction sites downstream of the Cλ7 exon by careful Southern blot analysis in single and double digests. The obtained detailed restriction map with the relevant restriction sites is given in Figure 1.

For each probe we carefully determined the germline restriction fragments for *Bgl*II, *Hin*dIII, *Bam*HI, and *Eco*RI (Table 2). The most optimal restriction enzymes for each "Igλ-isotype-specific" probe are summarized in Table 2 and some hybridization patterns are shown in Figure 2. Figure 3 shows the detection and identification of λ2 rearrangements by use of successive hybridization with the IGLC2D and ICLJ2 probes, respectively.

TABLE 2. Genomic probes and restriction enzymes for detection of human Igλ gene rearrangement.

DNA probe[a]	Restriction enzymes used for digestion of genomic DNA	Size of germline restriction fragments in kb	Allelic frequencies of RFLP's	Recommended restriction enzymes (preferential order)
IGLC1D	*Bgl*II	14.2	0% (0/114)	1. *Hin*dIII
	*Hin*dIII	9.0	0% (0/118)	2. *Bgl*II
IGLJ2	*Bgl*II	14.2 (Jλ2); 2.9 (Jλ3)	0% (0/116)	1. *Eco*RI-*Hin*dIII
	*Hin*dIII	9.0 (Jλ2); 10.1 (Jλ3) (and 11.6)[b]	0% (0/118)[d]	2. *Bgl*II
	*Bam*HI	0.7 (Jλ2); 0.7 (Jλ3)	0% (0/122)	3. *Sac*I
	*Eco*RI	8.4 (Jλ2 and Jλ3)	0% (0/146)[e]	
	*Eco*RI-*Hin*dIII	3.8 (Jλ2); 4.6 (Jλ3)	0% (0/112)[d]	
IGLC2D	*Eco*RI-*Hin*dIII	3.8 (Jλ2)	0% (0/112)[d]	*Eco*RI-*Hin*dIII
IGLC4D	*Bgl*II	3.8	0% (0/114)	1. *Bgl*II
	*Hin*dIII	10.1 (and 11.6)[b]	0% (0/118)	2. *Eco*RI-*Hin*dIII
	*Bam*HI	3.8	0% (0/120)	3. *Bam*HI-*Hin*dIII
	*Eco*RI	16.5 (and 28)[c]	0% (0/146)	
	*Eco*RI-*Hin*dIII	4.5 (and 5.9 kb)[b]	0% (0/118)	
	*Bam*HI-*Hin*dIII	2.7 (and 3.8 kb)[b]	0% (0/118)	
IGLJ5	*Bgl*II	5.8	0% (0/116)	1. *Bgl*II
	*Hin*dIII	14.5	0% (0/116)	2. *Eco*RI-*Hin*dIII
	*Bam*HI	0.7	0% (0/92)	
	*Eco*RI	16.5 (and 28)[c]	0% (0/146)	
	*Eco*RI-*Hin*dIII	10.5 (and 14.5)[c]	0% (0/116)	
IGLJ6	*Hin*dIII	14.5	0% (0/126)	1. *Bam*HI
	*Bam*HI	5.2	0% (0/126)	2. *Eco*RI-*Hin*dII
	*Eco*RI	16.5 (and 28)[c]	0% (0/146)	
	*Eco*RI-*Hin*dIII	10.5 (and 14.5)[c]	0% (0/126)	
IGLJ7	*Bgl*II	6.6	0% (0/120)	1. *Bam*HI
	*Hin*dIII	14.5	0% (0/128)	2. *Bgl*II
	*Bam*HI	2.7	0% (0/122)	3. *Eco*RI-*Hin*dIII
	*Eco*RI	16.5 (and 28)[c]	0% (0/146)	
	*Eco*RI-*Hin*dIII	10.5 (and 14.5)[b]	0% (0/128)	

a. The position of the DNA probes is indicated in Figure 1.
b. The *Hin*dIII germline restriction fragment of the J-Cλ4 gene region may vary in size due to partial digestion of the *Hin*dIII restriction site, which is located 1.2 kb downstream of the Cλ4 exon.
c. The *Eco*RI restriction site downstream of the Cλ7 gene segment is frequently resistant to digestion (8), resulting in an extra band of approximately 14.5 kb in *Eco*RI/*Hin*dIII digests and an extra band of approximately 28 kb in *Eco*RI digests, when hybridized with IGLC4D, IGLJ5, IGLJ6, or IGLJ7 DNA probes (Figure 1).
d. Except for and additional 5.4 kb band in case of polymorphic amplification of J-Cλ2/J-Cλ3 (allelic frequency: 20-30%) (15,16).
e. Except for additional 13.8 kb, 19.2 kb, and/or 24.6 kb bands in case of polymorphic amplification of J-Cλ2/J-Cλ3 (allelic frequency: 20-30%) (15,16).

Our extensive RFLP studies in a group of 73 healthy volunteers revealed no polymorphisms in the Igλ locus, except for the well-known genetic amplification polymorphism in the Cλ2- Cλ3 region, which gives an extension of ~5.4 kb for each

TABLE 3. Identification of "Igλ-isotype gene" rearrangements in 40 B-lineage malignancies.

	Total rearranged alleles	J-Cλ1	J-Cλ2	J-Cλ3	J-Cλ4	J-Cλ5	J-Cλ6	J-Cλ7
Precursor-B-ALL (n=20)	4	0	1	2	0	0	1	0
Igλ+ B-CLL (n=10)	13	2	4	6	0	0	0	1*
Igλ+ multiple myelomas (n=10)	15	1	8	6	0	0	0	0
TOTAL (n=40)	32	3 (9%)	13 (41%)	14 (44%)	0 (0%)	0 (0%)	1 (3%)	1 (3%)

* Lymph node from a B-cell lymphoma/B-CLL patient.

ımplification and has an allelic frequency of 20-30% in Caucasians (3,15,16). Also the :eported RFLP of the *Bam*HI restriction site in the J-Cλ3 intron (4), was not observed in ɔur study (Table 2). However, we occasionally found a *Hin*dIII site and an *Eco*RI site to ɔe partially resistant to digestion. The occasionally resistant *Hin*dIII restriction site is ocated 1.2 kb downstream of the Cλ4 exon, which results in an additional weak band ɔf 11.6 kb (5.9 kb in *Eco*RI/*Hin*dIII digests) upon hybridization with the IGLC4D probe Table 2). The occasionally resistant *Eco*RI site is located 1.4 kb downstream of Cλ7 :xon, which results in an additional weak band of approximately 28 kb (14.5 kb in *Eco*RI/*Hin*dIII digests), when hybridized with IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes Figure 2 and Table 2). Usage of the IGLJ5 probe in *Bgl*II digests resulted in a faint ıand of approximately 14 kb, which was not seen in other digests and could not be ttributed to underdigestion; therefore the origin of this faint band remains unexplained.

For optimal Southern blot analysis of Igλ gene rearrangements, we recently evaluated ıe use of a Cλ probe (IGLC3) in combination with *Eco*RI/*Hin*dIII double digestion, /hich allows detection of approximately 95% of all clonal Igλ gene rearrangements in ·-cell malignancies (8). However, this IGLC3 probe results in complex banding patterns nd is not suitable for identifying the various Igλ gene rearrangements. The here presented Igλ-isotype-specific" DNA probes allow easy detection and identification of :arrangements in the seven J-Cλ regions. Our preliminary analyses of a series of 40 ·-lineage malignancies (20 precursor-B-ALL, 10 Igλ+ B-CLL, and 10 Igλ+ multiple ıyelomas) demonstrated that indeed all clonal Igλ gene rearrangements could be lentified precisely (Table 3 and Figure 2). Rearrangements to the J-Cλ3 gene region ccurred most frequently (~45%), followed by J-Cλ2 (~40%) and J-Cλ1 (~10%) :arrangements, while rearrangements in the J-Cλ6 and J-Cλ7 regions were rare (Table). It should be noted that the Cλ6 exon is regarded to be a pseudogene due to a stop ɔdon (6). Nevertheless, rearrangement to the J-Cλ6 region can occur and can even :sult in expression of a truncated Igλ6 protein on the cell membrane (17).

We conclude that the here presented new set of DNA probes is useful for studies ı clonal "Igλ isotype" gene rearrangements in B-lineage malignancies.

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5

If I must choose between righteousness and peace, I choose righteousness
(T. Roosevelt)

CHAPTER 5

IMMUNOGLOBULIN LAMBDA ISOTYPE GENE REARRANGEMENTS IN B-CELL MALIGNANCIES*

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SUMMARY

The human immunoglobulin lambda (Igλ) locus contains seven J-Cλ gene regions, which are spread over a total distance of 33 kb. The J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 gene regions are functional and are assumed to encode for four distinct Igλ isotypes, i.e. Mcg, Ke^-Oz^-, Ke^-Oz^+, and Mcp, respectively, whereas the J-Cλ4, J-Cλ5, and J-Cλ6 regions are not functional.

To identify clonal Igλ gene rearrangements, we recently developed a new set of seven "isotype specific" DNA probes, which were used for detection of Igλ isotype gene rearrangements in 212 B-cell malignancies, i.e. 76 precursor-B-cell acute lymphoblastic leukemias (ALL), 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^+$ multiple myelomas. Rearrangements to the J-Cλ3 gene region occurred most frequently (~50%), followed by J-Cλ2 rearrangements (~40%) and J-Cλ1 rearrangements (~10%). Rearrangements to the J-Cλ6 and J-Cλ7 gene regions were rare and no rearrangements to J-Cλ4 and J-Cλ5 were detected. The latter is probably due to the inappropriate recombination signal sequences of the Jλ4 and Jλ5 gene segments. J-Cλ7 was rearranged in only one case out of 136 $Ig\lambda^+$ B-cell malignancies (0.7%), which is in accordance with the recent report by Niewold et al. (1996).

Interestingly, we observed differences in the occurrence of isotype rearrangements between the different types of $Ig\lambda^+$ B-cell malignancies. Chronic B-cell leukemias and B-NHL contained J-Cλ2 rearrangements in 32% of cases and J-Cλ3 rearrangements in 54%, whereas multiple myelomas showed frequent rearrangements to the J-Cλ2 region (60%), followed by J-Cλ3 rearrangements (37%). This significantly differential occurrence of J-Cλ2/J-Cλ3 isotype rearrangements seems to be dependent on the maturation stage of the B-cell malignancy and might therefore be caused by selection processes.

INTRODUCTION

The human immunoglobulin lambda (Igλ) light chain gene has been mapped to chromosome 22q11 and seven J-Cλ gene regions have been characterized over a total distance of 33 kb (1-6). The gene regions J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7 are functional, while J-Cλ4, J-Cλ5, and J-Cλ6 are non-functional (pseudo) gene regions due to frame shifts (4, 7, 8).

So far, studies concerning Igλ isotype usage are based on amino acid sequencing of various Bence-Jones proteins and Igλ chains isolated from intact immunoglobulins (9, 10). Initially, four different isotypes of Igλ light chains were identified, Mcg, Ke^-Oz^-, Ke^-Oz^+, and Ke^+Oz^- (1, 8, 9, 11), which were assumed to be encoded by the four functional J-Cλ gene regions, J-Cλ1, J-Cλ2, J-Cλ3, and J-Cλ7, respectively. However, the amino acid sequence of the Ke^+Oz^- isotype differed from the J-Cλ7 nucleotide sequence at five amino acid positions (4, 8). This is supported by the recent study of Niewold et al., proving that the newly discovered Mcp isotype is encoded by the J-Cλ7 gene segment (12). It is now assumed that the Ke^+Oz^- isotype is encoded by a polymorphic J-Cλ2 gene segment or a duplicated J-Cλ2 gene segment (8, 12).

Because at least one third of all B-cell malignancies have rearranged their Igλ genes, we developed J-Cλ gene region specific probes to detect and identify the clonal Igλ gene rearrangements in these malignancies (13). These J-Cλ "isotype specific" DNA probes were used to determine the frequency of the J-Cλ gene rearrangements in a large series of 212 B-cell malignancies, i.e. 76 precursor-B-cell acute lymphoblastic leukemias (precursor-B-ALL), 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^+$ multiple myelomas. The results of the J-Cλ rearrangement patterns are compared with the reported frequencies of Igλ isotype protein expression (9, 10). We are aware that the J-Cλ "isotype gene rearrangements" at the DNA level do not necessarily reflect Igλ protein expression, especially in B-cell malignancies with biallelic Igλ gene rearrangements. Nevertheless we assume that the Igλ isotype rearrangement patterns in the various types of B-cell malignancies will give insight into Igλ isotype usage and/or Igλ isotype selection during B-cell differentiation.

MATERIALS AND METHODS

Cell samples

We selected a large series of B-cell malignancies from our cell bank, based on the availability of sufficient cells for DNA extraction, the expression of Igλ light chains in case of mature Ig^+ B-cell malignancies, and based on a high frequency of tumor cells, i.e. >70% in case of leukemias and lymphomas and >25% in case of multiple myelomas. In this way 117 peripheral blood (PB), 78 bone marrow (BM), and 17 lymph node samples were selected from 212 patients with a B-cell malignancy, i.e. 76 precursor-B-ALL, 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-NHL, and 28 $Ig\lambda^+$ multiple myelomas. The series of 74 chronic B-cell leukemias consisted of 63 B-cell chronic lymphocytic leukemias (B-CLL), 6 B-cell prolymphocytic leukemias (B-PLL), and 5 hairy cell leukemias (HCL). In case of PB and BM samples, mononuclear cells were isolated by Ficoll-Paque density centrifugation (density: 1,077 g/l; Pharmacia, Uppsala, Sweden). Cell line Hela without Igλ gene rearrangements was used as negative control (5).

Southern blot analysis

DNA was isolated as described previously (5, 14). Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia). The restriction fragments were size-fractioned in 0.7% agarose gels and transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) (5). The membranes were hybridized with ^{32}P-random oligonucleotide labeled probes (5).

Igλ DNA probes and restriction map

In order to identify clonal gene rearrangements in the J-Cλ gene locus, we recently developed a set of seven isotype-specific DNA probes, which specifically recognize the seven J-Cλ regions: the IGLC1D probe for the J-Cλ1 gene region, the IGLJ2 probe for the highly homologous J-Cλ2 and J-Cλ3 gene regions, the IGLC2D probe which exclusively recognizes the J-Cλ2 gene region, and the IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes for the last four J-Cλ gene regions, respectively (13). These new isotype-specific DNA probes were used in combination with *Hin*dIII, *Bgl*II, *Bam*HI/*Hin*dIII, *Eco*RI/*Hin*dIII restriction enzyme digests. The restriction map of the J-Cλ gene region is given in Figure 1 (4, 8, 13). In some cases *Sac*I digests provided additional information for discrimination between J-Cλ2 and J-Cλ3 rearrangements with the IGLJ2 probe.

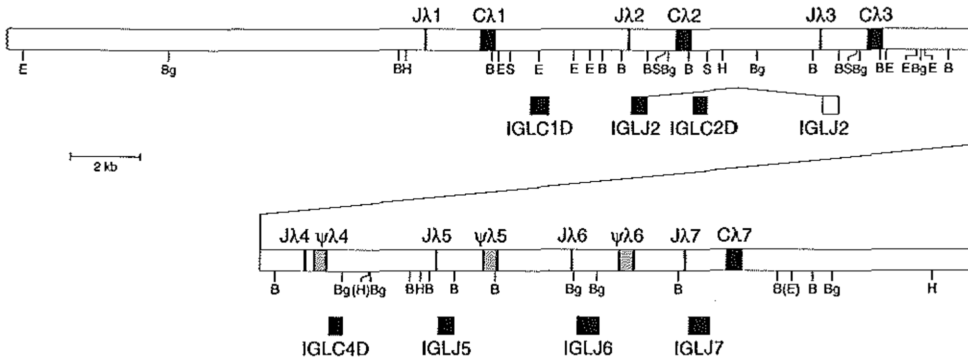

Figure 1. Structure and restriction map of the J-C region of the human Igλ gene complex (4, 8, 13). The location of the relevant *Hin*dIII (H), *Bgl*II (Bg), *Bam*HI (B), and *Eco*RI (E) sites are indicated; the relevant *Sac*I sites are only indicated for the J-Cλ2 and J-Cλ3 gene regions (4). The partially resistant *Hin*dIII and *Eco*RI restriction sites downstream of the ψCλ4 and Cλ7 exons, respectively, are in parentheses. The solid boxes represent the functional Cλ exons and the dotted boxes represent the non-functional (pseudo; ψ) Cλ exons. The probes are indicated as solid bars. These probes are specific for the corresponding J-Cλ gene segments, except for the IGLJ2 probe, which recognizes both J-Cλ2 and J-Cλ3 gene regions due to a high homology of 98%. The recognition site of the IGLJ2 probe in the J-Cλ3 region is indicated with an open bar.

RESULTS AND DISCUSSION

About 40% of all B-cell malignancies have rearranged their Igλ genes, which therefore can be used for clonality studies (15). Despite this relatively high frequency of Igλ gene rearrangements, Igλ genes are rarely studied in B-cell malignancies because of the complex structure of the human Igλ locus. This is the first detailed study on Igλ isotype gene rearrangements in a large series of 212 B-cell malignancies. All analyzed 136 B-cell malignancies with Igλ protein expression had at least one rearranged Igλ allele; in 35% (47/136) of cases both Igλ alleles were rearranged, and one case with B-

TABLE 1. Igλ isotype gene rearrangements in 212 B-cell malignancies.

B-cell malignancies	J-Cλ1	J-Cλ2	J-Cλ3	J-Cλ4	J-Cλ5	J-Cλ6	J-Cλ7	Unidentified rearrangement	Total number of rearranged alleles
Igλ+ chronic B-cell leukemia (n=74)	10 (10%)	32 (33%)	53 (55%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (1%)	96
Igλ+ B-NHL (n=34)	7 (13%)	16 (30%)	27 (51%)	0 (0%)	0 (0%)	0 (0%)	1 (2%)	1 (2%)	52
Igλ+ multiple myeloma (n=28)	1 (3%)	21 (60%)	13 (37%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	35
Total Igλ+ malignancies (n=136)	18 (10%)	69 (38%)	93 (51%)	0 (0%)	0 (0%)	0 (0%)	1 (0.5%)	2 (1%)	183
precursor-B-ALL (n=76)	0 (0%)	9 (43%)	8 (38%)	0 (0%)	0 (0%)	4 (19%)	0 (0%)	0 (0%)	21
Total B-cell malignancies (n=212)	18 (9%)	78 (38%)	101 (50%)	0 (0%)	0 (0%)	4 (2%)	1 (0.5%)	2 (1%)	204

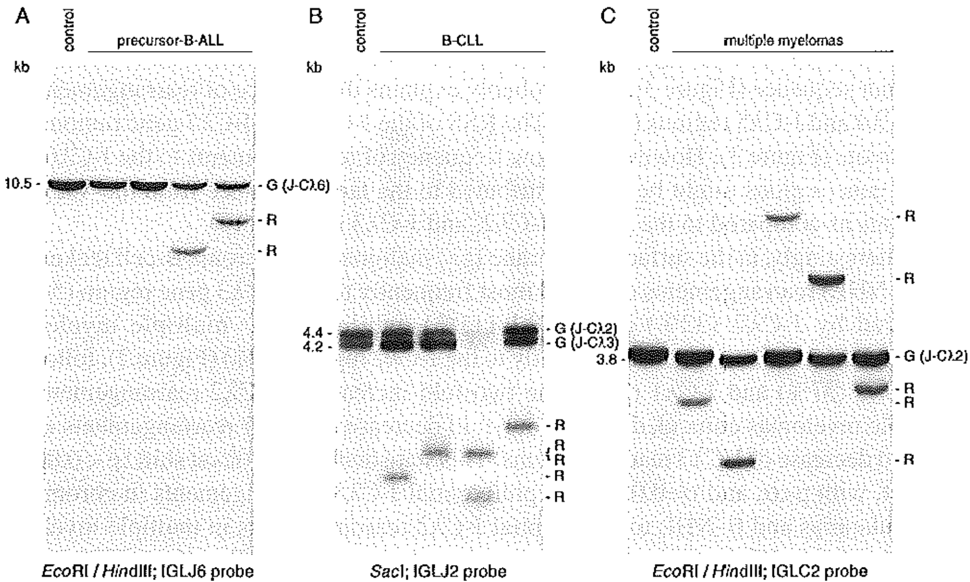

Figure 2. Southern blot analysis of Igλ genes in B-cell malignancies. DNA samples from control DNA, precursor-B-ALL patients (A), B-CLL patients (B), and multiple myeloma patients (C) were digested with *Eco*RI/*Hin*dIII or *Sac*I, size fractionated in agarose gels, blotted onto a nylon membrane, and hybridized with ^{32}P-labeled DNA probes. A: This panel illustrates that J-Cλ6 gene rearrangements can occur in precursor-B-ALL. In contrast, in Igλ^{+} B-cell malignancies no rearrangements to J-Cλ6 were detected. B: Hybridization of a *Sac*I filter with the IGLJ2 probe can provide additional information for discrimination between J-Cλ2 and J-Cλ3 gene rearrangements. Rearrangement to the J-Cλ2 region leaves the J-Cλ3 region in germline configuration: the J-Cλ3 band (4.2kb) appears as more dense. C: This panel illustrates the use of the IGLCD2 probe for easy detection of J-Cλ2 rearrangements, which are frequently found in multiple myelomas.

CLL had three rearranged alleles. In contrast, only 20% (15/76) of precursor-B-ALL had rearranged Igλ genes: ten cases had one, four cases had two, and one case had three rearranged alleles. This low frequency of Igλ gene rearrangements in precursor-B-ALL is in line with the immature differentiation stage of this type of leukemia (15). Nevertheless, the observed frequency of ~20% is essentially higher than the ~5% (5/92) as reported in the literature (reviewed in ref. 15), but is comparable to the ~23% (14/60) in our previous study (16). Apparently our DNA probes are more efficient in detecting Igλ gene rearrangements (Figure 2).

Most rearrangements in our series of 212 B-cell malignancies occurred to J-Cλ3 (~50%), followed by J-Cλ2 (~40%) and J-Cλ1 (~10%), while rearrangements to J-Cλ6 and J-Cλ7 were rare (Table 1). Rearrangements to J-Cλ4 and J-Cλ5 were not identified (Table 1). In two cases the Igλ rearrangements could not be identified precisely, which might be caused by a chromosome aberration. Differences in the usage of the J-Cλ1, J-Cλ2, and J-Cλ3 regions can not be explained by more or less optimal recombination signal sequences (RSS) (Figure 3). The three bases of the RSS heptamer sequence closest to the recombination cleavage site and the sixth and seventh nucleotides of the RSS nonamer sequence are critical for V(D)J recombination (17,18). These RSS

nucleotide positions are identical for the Jλ1, Jλ2, and Jλ3 gene segments (Figure 3). However, the Jλ4 and Jλ5 gene segments do not contain all essential nucleotides of the heptamer sequence (Figure 3). This probably explains the absence of rearrangements to the J-Cλ4 and J-Cλ5 regions (17,18). The RSS of the J-Cλ6 and J-Cλ7 gene regions are appropriate (Figure 3). However, the low frequency of rearrangements to the J-Cλ6 and J-Cλ7 gene regions might be partly attributed to the larger distance from the Vλ gene segments as compared to the J-Cλ1, J-Cλ2, and J-Cλ3 regions (19).

Interestingly, in the surface membrane Igλ$^+$ malignancies (chronic B-cell leukemias and B-NHL) rearrangements occurred most frequently to the J-Cλ3 gene region (54%), followed by J-Cλ2 (32%) and J-Cλ1 (11%), while in Igλ$^+$ multiple myelomas rearrangements occurred most frequently to the J-Cλ2 region (60%) (Figure 2), followed by J-Cλ3 (37%) and J-Cλ1 (3%). The two-fold increase of J-Cλ2 rearrangements in multiple myelomas coincided with a four-fold decrease of J-Cλ1 rearrangements and a 1.5 fold decrease of J-Cλ3 rearrangements. This statistically significant shift ($p < 0.0064$) (χ^2 test, with $p<\alpha$, and $\alpha=0.05$) from preferential J-Cλ3 rearrangements in chronic B-cell leukemias and B-NHL to preferential J-Cλ2 rearrangements in multiple myeloma might be due to Igλ isotype selection processes during terminal B-cell maturation, e.g. related to somatic mutations or Ig heavy (IgH) chain class switch. Theoretically, preferential pairing of particular IgH and Ig light (IgL) chains might determine preferential pairing of Cλ3 chains with Igμ chains in chronic B-cell leukemias and B-NHL, and Cλ2 chains with Igγ and Igα chains in multiple myeloma. However, so far no such IgH class-dependent preferential pairing mechanisms have been described.

The distribution of Igλ isotype gene rearrangements in multiple myeloma indeed resembled the reported Igλ isotype pattern of Bence Jones proteins: Cλ2 (Ke$^-$Oz$^-$) occurred most frequently, followed by Cλ1 (Mcg), Cλ3 (Ke$^-$Oz$^+$), and Cλ7 (Mcp) (Table 2) (9, 10, 12). However, in the Bence Jones protein studies the frequency of Cλ1 (Mcg) expression appeared to be essentially higher (23%) than the frequency of J-Cλ1 isotype rearrangements in multiple myeloma (3%) (Table 2). This discrepancy is difficult to

	Heptamer	Spacer	Nonamer
Consensus RSS	**CACAGTG**	**12 bp**	**ACAAAAACC**
Jλ1 RSS	-------	12	--C------
Jλ2 RSS	---T---	12	---------
Jλ3 RSS	---T---	12	---------
Jλ4 RSS	TG-G---	12	--------T
Jλ5 RSS	TG-T---	12	---------
Jλ6 RSS	---T---	12	---C-----
Jλ7 RSS	-------	12	---C-----

Figure 3. Consensus RSS and the RSS of the seven Jλ gene segments are shown. The consensus heptamer and nonamer are shown in bold letters. The underlined nucleotides are crucial for optimal function of the RSS (17, 18). Only the mismatching nucleotides of the Jλ RSS are indicated.

TABLE 2. Correlation between Igλ isotype protein expression and the Igλ isotype gene rearrangement in multiple myeloma.

Multiple myeloma	J-Cλ1 (Mcg)	J-Cλ2 (Ke^-Oz^-)	"polymorphic J-Cλ2" (Ke^+Oz^-)	J-Cλ3 (Ke^-Oz^+)	J-Cλ7 (Mcp)
Igλ isotype protein expression (n=70)[a]	16/70 (23%)	36/70 (51%)	4/70 (6%)	14/70 (20%)	<1%[b]
Igλ isotype gene rearrangements (n=35); this study	1/35 (3%)	21/35[c] (60%)		13/35 (37%)	0/35 (0%)

a. Based on literature data by Fett and Deutsch (9) and Walker et al. (10). The number of studied Bence Jones proteins in the study by Walker et al. (10) is corrected for duplicates with the study by Fett and Deutsch (9).
b. According to Niewold et al. the frequency of the Mcp isotype is <1% (12).
c. The IGLJ2 and IGLC2D probes cannot discriminate between rearrangements in normal and polymorphic J-Cλ2 regions.

explain; it might be that the studied series of Bence Jones proteins was a non-random selection or it might be that formation of Bence Jones proteins is influenced by the isotype of the Igλ chain.

In our analyses of 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-NHL, and 28 $Ig\lambda^+$ multiple myelomas only one B-NHL patient contained a rearrangement to the J-Cλ7 gene region. This is in line with the recent report by Niewold et al., who demonstrated that the J-Cλ7 region encodes for the rarely occurring Mcp isotype (12). Thereby Niewold et al. also have proven that the Ke^+Oz^- isotype is not encoded by the J-Cλ7 region. The Ke^+Oz^- isotype is now assumed to be encoded by a polymorphic J-Cλ2 region, e.g. the amplified J-Cλ2 region (8, 12). Our Igλ-isotype specific DNA probes can not discriminate between rearrangements in normal J-Cλ2 regions and polymorphic J-Cλ2 regions. However, the reported combined frequency of Ke^-Oz^- and Ke^+Oz^- myeloma proteins (57%) is comparable to our frequency of J-Cλ2 rearrangements (60%) in multiple myelomas (Table 2).

Curiously, the four detected J-Cλ6 rearrangements were exclusively found in precursor-B-ALL and represented ~20% of all Igλ rearrangements in this group of B-cell malignancies (Figure 2). The absence of J-Cλ6 rearrangements in mature $Ig\lambda^+$ B-cell malignancies can be explained by the fact that the J-Cλ6 region can only encode for a truncated protein, not for a complete Igλ chain (20).

Here we present the first report on the Igλ isotype gene rearrangements in a large series of B-cell malignancies. We conclude from this study that in B-cell malignancies the Igλ isotype rearrangements to J-Cλ3 occur in ~50% of cases, followed by J-Cλ2 in ~40%, and J-Cλ1 in ~10%, while rearrangements to J-Cλ6 and J-Cλ7 are rare. We also found significant differences in Igλ isotype gene rearrangements between chronic B-cell leukemias and B-NHL as compared to multiple myelomas, which indicates that Igλ isotype selection processes might occur during B-cell maturation.

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6

A man's usefullness depends upon his ideals, insofar as he can. It's hard to fail, but it's worse never to have tried to succeed (T. Roosevelt)

CHAPTER 6

EASY DETECTION AND IDENTIFICATION OF IMMUNOGLOBULIN LAMBDA GENE REARRANGEMENTS BY CONFINED SOUTHERN BLOT ANALYSIS*

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SUMMARY

Previous experiments revealed that Southern blot analysis of Igλ gene rearrangements with a general Cλ3 probe (IGLC3) in combined *Eco*RI/*Hin*dIII digests allows detection of approximately 95% of all Igλ gene rearrangements. This probe-enzyme combination is reasonably efficient in B-cell malignancies with a high tumor load (>75%), but results in complex banding patterns of multiple germline and rearranged bands of different density. This might cause detection problems in case of low tumor load (<75%). Especially rearrangements in the J-Cλ1 region can be missed. More detailed analysis of rearrangements to the seven J-Cλ regions of the Igλ locus appeared to be possible with our seven Igλ isotype specific probes and allowed sensitive detection and identification of 100% and 99% of the Igλ gene rearrangements, respectively. The disadvantage of this approach is the extensive number of hybridization steps (approximately 20 hybridizations). Extensive Southern blot analysis with the seven Igλ isotype specific DNA probes in a large series of 212 B-cell malignancies revealed that 97% (197/204) of all Igλ rearrangements occurred to the J-Cλ1, J-Cλ2, or J-Cλ3 gene regions. Therefore, it should be possible to detect and identify virtually all Igλ rearrangements using a limited set of Igλ isotype specific probes. We compared the effectiveness of the IGLC1D, IGLJ2, and IGLC2D isotype probes with the general IGLC3 probe and the total set of seven Igλ isotype probes. Based on this comparative study we conclude that rearrangements in the J-Cλ1, J-Cλ2, and J-Cλ3 gene regions can be detected with three appropriate isotype specific DNA probes in *Bgl*II, *Hin*dIII, and *Eco*RI/*Hin*dIII digests (5 hybridizations). This approach allowed sensitive detection and identification of 96% (195/204) of all Igλ gene rearrangements. Further restriction of Southern blot analysis to the use of the IGLC1D and IGLJ2 probes in *Bgl*II digests (2 hybridizations) still allowed detection of 94% (192/204) of all Igλ gene rearrangements, but discrimination between J-Cλ2 and J-Cλ3 gene rearrangements was difficult in cases with low tumor load.

INTRODUCTION

Southern blot analysis with a general Igλ probe (IGLC3) in *Eco*RI/*Hin*dIII digests allows detection of 95% of all Igλ rearrangements (1). This probe-enzyme combination works reasonably well as long as the tumor load is high. However, in case of lower tumor loads (<75%), detection of rearranged bands is less efficient due to faint signals. Especially rearrangements to the J-Cλ1 region can be missed.

For identification of rearrangements to the seven J-Cλ regions of the Igλ locus we recently developed seven Igλ "isotype specific" DNA probes (2). These probes appeared to be highly specific, resulting in easy detection of germline and rearranged bands (2). Furthermore, the probes are more sensitive than the IGLC3 probe and allow routine detection of low tumor loads, down to approximately 5%. The seven Igλ isotype specific probes were used for detailed analysis of the Igλ gene rearrangement patterns in a large series of 212 B-lineage malignancies, i.e. 76 precursor-B-acute lymphoblastic leukemia (ALL), 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^+$ multiple myelomas (3). This extensive study demonstrated that the vast majority (97%) of all Igλ rearrangements occurred to the J-Cλ1, J-Cλ2, or J-Cλ3 gene regions.

However, the usage of seven probes in two to four restriction enzyme digests per probe (a total of approximately 20 hybridizations) is time consuming and labour-intensive. Therefore we evaluated whether a limited set of Igλ isotype probes can be used for detection and identification of virtually all Igλ gene rearrangements with a high sensitivity.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB), bone marrow (BM), or lymph node samples of a large series of 212 patients with B-lineage malignancies, i.e. 76 precursor-B-ALL, 74 $Ig\lambda^+$ chronic B-cell leukemias, 34 $Ig\lambda^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 $Ig\lambda^+$ multiple myelomas were used (3). Mononuclear cells (MNC) were isolated from PB and BM samples by Ficoll-Paque centrifugation (density: 1,077 g/l; Pharmacia, Uppsala, Sweden). DNA was extracted from MNC and lymph node cells for Southern blot analysis (1,4). DNA from the cell line Hela without Igλ rearrangements was used as germline control (4).

Southern blot analysis

Fifteen micrograms of DNA were digested with the appropriate restriction enzymes (Pharmacia), size-fractioned in 0.7% agarose gels, transferred by vacuum blotting to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany), and hybridized with ^{32}P-random oligonucleotide labeled probes (4,5).

Restriction map and Igλ DNA probes

To detect and identify the Igλ gene rearrangements, we previously determined the precise restriction map of the human J-Cλ locus and constructed the general Igλ probe (IGLC3) and the seven Igλ isotype specific DNA probes (Figure 1) (1,2,6).

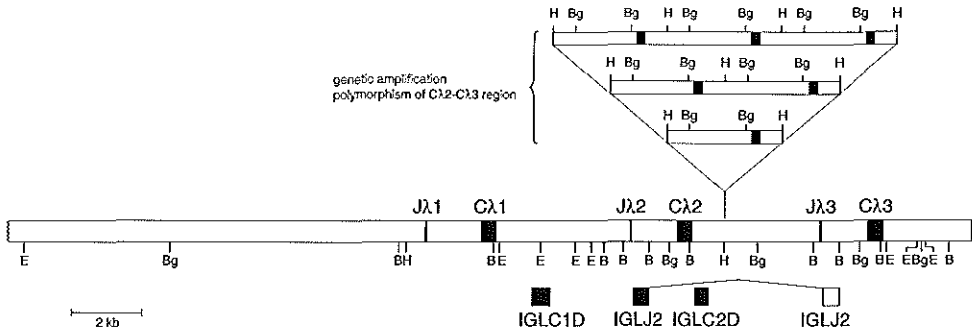

Figure 1. Structure and restriction map of the first three J-Cλ gene regions of the human Igλ gene complex, including the genetic amplification polymorphism (P1, P2, and P3) of the J-Cλ2 gene region. The location of the relevant *Hin*dIII (H), *Bgl*II (Bg), and *Eco*RI (E) are indicated. The solid boxes represent the most frequently used Cλ exons and the probes are indicated as solid bars. The IGLC1D probe recognizes only the J-Cλ1 gene region and the IGLC2D recognizes exclusively J-Cλ2 gene region, while the IGLJ2 probe recognizes both J-Cλ2 and J-Cλ3 gene regions due to a high homology (98%).

RESULTS AND DISCUSSION

For our diagnostic clonality studies, we developed a general Cλ probe (IGLC3) which allows detection of 95% of all Igλ gene rearrangements in *Eco*RI/*Hin*dIII digests (1). In routine diagnostics this single probe-enzyme combination works reasonably well, provided the frequency of clonal B-cells is high enough (>75%). However, complex banding patterns are obtained with multiple germline bands of different density, which need experience for correct interpretation (Figure 2A). This is caused by hybridization of the general Cλ probe with the various Cλ gene segments of the classical and surrogate λ-like loci, which show different degrees of homology to the IGLC3 probe (1,6). For the same reason also the rearranged bands might have a low density despite a high tumor load. In practice this especially concerns weak rearranged bands in case of J-Cλ1 gene rearrangements, due to the reduced (93%) homology between the Cλ1 gene segment and the IGLC3 probe (Figure 2) (1,2,6). Furthermore, comigration of rearranged bands with one of the multiple germline bands might occur, which probably explains that ~5% of the Igλ gene rearrangements remain undetected with the IGLC3 probe in *Eco*RI/*Hin*dIII digests (1).

To improve the detectability and identification of Igλ gene rearrangements, we developed a set of seven Igλ isotype specific DNA probes: the IGLC1D probe for the J-Cλ1 gene region, the IGLC2D probe for the J-Cλ2 gene region, IGLJ2 for the highly homologous J-Cλ2 and J-Cλ3 gene regions, and the IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes for the four remaining J-Cλ gene regions (2). Due to their specificity and the absence of cross-hybridizations to other Igλ gene segments, these probes have the same sensitivity as other optimal Southern blot probes, approximately 5%, and do not result in multiple germline bands (Figure 2).

The seven isotype specific probes were applied in approximately 20 probe-enzyme combinations for detailed analysis of 212 B-cell malignancies: 76 precursor-B-ALL, 74

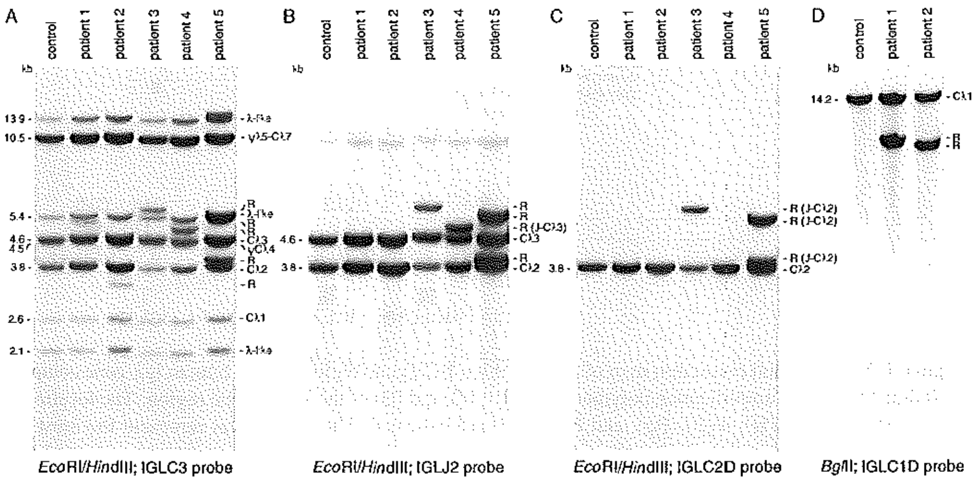

Figure 2. Comparative Southern blot analysis in B-cell malignancies. Control DNA, and DNA from five Igλ⁺ malignancies were digested with *Eco*RI/*Hin*dIII or *Bgl*II, size fractionated in agarose gels, blotted onto nylon membrane, and hybridized with ^{32}P-labeled IGLC3, IGLJ2, IGLC2D, IGLC1D probes. **A:** This panel illustrates that detection of Igλ gene rearrangements with IGLC3 probe in combined *Eco*RI/*Hin*dIII digest is efficient, but results in multiple germline bands and rearranged bands of different density. Panel **B** illustrates that most rearranged bands occurred to either J-Cλ2 or J-Cλ3 gene regions. **C:** This panel illustrates that the IGLC2D probe clearly identifies rearrangements to the J-Cλ2 gene region. The germline and rearranged J-Cλ3 bands (as seen in panel B) are absent. **D:** This panel illustrates that the two rearrangements to the J-Cλ1 gene region can clearly be identified with the IGLC1D probe. It should be noted that J-Cλ1 gene rearrangements frequently give a faint band if hybridized with the general IGLC3 probe (panel A, lanes 2 and 3) because of the lower homology between the Cλ1 exon and the other Cλ exons.

Igλ⁺ chronic B-cell leukemias, 34 Igλ⁺ B-NHL, and 28 Igλ⁺ multiple myelomas (3). This extensive Southern blot study demonstrated that 97% (197/204) of the rearrangements occurred to the J-Cλ1, J-Cλ2, and J-Cλ3 gene regions, while rearrangements to the remaining J-Cλ gene regions were rare (2%, 5/204); two rearrangements (1%, 2/204) remained unidentified (3).

To reduce the high number of hybridizations, we evaluated whether Igλ gene analyses can be restricted to the first three J-Cλ gene regions. For this purpose, we firstly determined to what extent the general Cλ probe (IGLC3) detected all Igλ gene rearrangements. Next, we evaluated whether the restricted analysis of the first three gene regions with specific DNA probes (IGLC1D, IGLC2D, and IGLJ2) in *Eco*RI/*Hin*dIII, *Bgl*II, and/or *Hin*dIII digests could detect and identify the rearrangements in the corresponding J-Cλ regions and to what extent this approach could replace the application of all seven Igλ isotype specific probes.

Table 1 shows that application of the IGLC3 probe in *Eco*RI/*Hin*dIII digests allowed detection of Igλ gene rearrangements in 97% of the 212 B-cell malignancies and in 94 % of the rearranged alleles. Furthermore, the application of all seven Igλ isotype probes in the appropriate digests allowed detection and identification of 100% and 99% of all rearranged Igλ alleles, respectively (Table 1). The restricted application of the relevant

TABLE 1. Comparison of three probe sets for detection of human Igλ gene rearrangements.

B-cell malignancies[a]	IGLC3 probe (one hybridization)		All seven isotype probes[b] (twenty hybridizations)			IGLC1D, IGLC2D, and IGLJ2 probes (five hybridizations)	
	Patients	Alleles	Patients	Alleles		Patients	Alleles
				identified	unidentified		
Igλ+ CBL (n=74)	70/74 (95%)	89/96 (93%)	74/74 (100%)	95/96 (99%)	1/96 (1%)	72/74 (97%)	93/96 (97%)
Igλ+ B-NHL (n=34)	34/34 (100%)	50/52 (96%)	34/34 (100%)	51/52 (98%)	1/52 (2%)	34/34 (100%)	50/52 (96%)
Igλ+ MM (n=28)	28/28 (100%)	34/35 (97%)	28/28 (100%)	35/35 (100%)	0/35 (0%)	28/28 (100%)	35/35 (100%)
Subtotal (n=136)	132/136 (97%)	173/183 (95%)	136/136 (100%)	181/183 (99%)	2/183 (1%)	134/136 (99%)	178/183 (97%)
Prec. B-ALL (n=76)	14/15 (93%)	19/21 (90%)	15/15 (100%)	21/21 (100%)	0/21 (0%)	11/15 (73%)	17/21 (81%)
Total (n=212)	146/151 (97%)	192/204 (94%)	151/151 (100%)	202/204 (99%)	2/204 (1%)	145/151 (96%)	195/204 (96%)

a. Abbreviations: CBL = chronic B cell leukemias; B-NHL = B cell non-Hodgkin lymphomas; MM = multiple myelomas; Prec. B-ALL = precursor-B-ALL.

b. We assume that analysis with the seven isotype specific probes detects all Igλ gene rearrangements (3).

c. Only 15 of the 76 precursor-B-ALL (20%) contained Igλ gene rearrangements. A total of 21 rearranged alleles was found in these 15 patients (see ref. 3 for details).

IGLC1D, IGLC2D, and IGLJ2 probes in the appropriate digests appeared to be highly informative: first round hybridization of the IGLC2D probe to *Eco*RI/*Hin*dIII filters and the IGLC1D probe to *Bgl*II and *Hin*dIII filters and second round hybridization of the IGLJ2 probe to *Eco*RI/*Hin*dIII and *Bgl*II filters in B-cell malignancies allowed detection and identification of 96% of all Igλ gene rearrangements (Table 1, Figure 2). Only in precursor-B-ALL approximately 20% of the rearrangements were missed (Table 1), because these concerned J-Cλ6 rearrangements, which were not found in $Ig\lambda^+$ B-cell malignancies (3).

In conclusion, instead of 20 probe-enzyme combinations, it appeared to be possible to efficiently detect and identify 96% of all Igλ gene rearrangements by using only three probes in two or three digests (5 hybridizations). These analyses are easy to perform and to interpret and have the same sensitivity (~5%) as other well-designed DNA probes (Figure 2). In case of limited amounts of DNA, one might even decide to restrict the analyses to a single *Bgl*II digest and successive hybridization with the IGLJ2 probe (for the J-Cλ2 and J-Cλ3 regions) and the IGLC1D probe (for the J-Cλ1 region). This approach allowed detection of 94% (192/204) of all Igλ gene rearrangements (data not shown), but discrimination between J-Cλ2 and J-Cλ3 rearrangements was difficult if the tumor load was not high (<75%). The advantage of this approach is that the same *Bgl*II filter can also be used for analysis of the Ig heavy chain (IgH) gene with the IGHJ6 probe and for analysis of the Igκ gene with the IGKJ5 and IGKDE probes (7,8). Generally, we advice to use two restriction enzyme digests per probe for reliable Southern blot studies (9). Nevertheless, our experience with diagnostic clonality studies in more than thousand samples indicate that *Bgl*II digests are informative in the vast majority of cases (>95%) and rarely show restriction fragment length polymorphisms in the relevant IgH, Igκ, and Igλ gene regions (2,4,7,8).

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7

Docendo discimus (Seneca, Epist. 7,7)
Door te onderwijzen, leren wij zelf

CHAPTER 7

GENERAL DISCUSSION

Until recently optimally chosen DNA probes for reliable Southern blot analysis of immunoglobulin (Ig) genes were only available for Ig heavy chain (IgH) genes and Ig kappa (Igκ) genes (1,2). In the here described study, we developed a general Cλ probe and seven Igλ isotype specific DNA probes and carefully selected optimal restriction enzymes for each probe, allowing the detection and the identification of the various rearranged Igλ genes (3,4). Therefore, now the tools for analysis of all three human Ig gene loci are available. These tools allow us to perform diagnostic clonality studies in all types of B-cell malignancies, to identify the Ig gene rearrangement patterns in the various B-cell malignancies, to study the ordered Ig gene rearrangement processes during B-cell differentiation, and to unravel allelic exclusion of Ig light chain (IgL) genes, i.e. the phenomenon that B-cells express only a single type IgL chain on the surface membrane.

Identification of Igλ gene rearrangements in B-cell malignancies

We first developed the general Igλ probe IGLC3 that allows detection of 95% of all Igλ rearrangements in clonal B-cell malignancies, if combined with *Eco*RI/*Hin*dIII digests (Chapter 3). In order to identify the various Igλ isotype gene rearrangements, we subsequently developed seven Igλ isotype specific DNA probes (Chapter 4). Our extensive study in 212 B-cell malignancies demonstrated that these probes allow detection and identification of 100% and 99% of all Igλ gene rearrangements, respectively (Chapter 5). Furthermore, the Igλ isotype rearrangement patterns in the various types of B-cell malignancies showed that in chronic B-cell leukemias (CBL), which are regarded as pre-follicular malignancies, most rearrangements occurred to the J-Cλ3 gene region (~55%), while in multiple myelomas, regarded as fully differentiated post-follicular malignancies, most rearrangements occurred to the J-Cλ2 gene region (60%) (Chapter 5). This shift from preferential rearrangements to the J-Cλ3 region in pre-follicular differentiation stages to rearrangements to the J-Cλ2 region in post-follicular differentiation stages might be caused by selection processes, e.g. preferential pairing of Igλ3 chains with Igμ chains and preferential pairing of Igλ2 chains with Igγ chains or other IgH chains. The Igλ gene rearrangement patterns in non-Hodgkin lymphomas appeared to be closer to CBL than to multiple myelomas, because of the higher frequency of Jλ3 rearrangements (51%) compared to Jλ2 rearrangements (30%). In Igλ$^+$ B-cell malignancies no rearrangements to pseudo J-Cλ regions (J-Cλ4, J-Cλ5, or J-Cλ6) were

observed, but in precursor-B-ALL rearrangements to the pseudo J-Cλ6 gene region were detected in ~20% of the Igλ rearrangements (Chapter 5). Despite the presence of J-Cλ6 rearrangements in precursor-B-ALL, mature B-cell malignancies apparently lack these rearrangements, probably because no functional J-Cλ6 protein can be produced (Chapter 5) (5). The complete absence of rearrangements to the pseudo gene regions J-Cλ4 and J-Cλ5 in precursor-B-ALL and Igλ$^+$ B-cell malignancies is probably caused by the inappropriate recombination signal sequences (RSS) of these two gene regions.

Interestingly, rearrangements to the J-Cλ7 gene region occurred only once (<1%) in the total group of analyzed B-cell malignancies, which represented most B-cell differentiation stages. This is in line with the recent data of Niewold et al., who showed that the J-Cλ7 gene region encodes the newly defined Mcp Igλ isotype, which is rarely expressed (<1%) (6).

Ordered or stochastic IgL gene rearrangement processes?

In normal and malignant human B-cells functional expression of Igκ genes occurs more frequently than functional expression of Igλ genes, resulting in a Igκ/Igλ distribution of approximately 1.4. Two models have been proposed to explain this relative 'over usage' of Igκ genes: the ordered and the stochastic model (7-11). The first model argues that Igκ genes rearrange prior to Igλ genes, because the Igκ gene is extensively 'used' in Igλ$^+$ B-cells, while most Igκ$^+$ B-cells have germline Igλ genes (2,12). The stochastic model argues that both IgL genes rearrange totally independent, but that other factors handicap Igλ gene rearrangements, such as inefficient RSS (13), the complex structure of the human Igλ genes with separated J gene segments (14,15), and a lower number of V gene segments in the Igλ locus than in the Igκ locus (13).

To determine which model is applicable to the human IgL gene rearrangements, information is needed about the configuration of both Igκ alleles and both Igλ alleles at 'the single B-cell level'. For this purpose we used a series of 105 CBL: 53 Igκ$^+$ CBL and 52 Igλ$^+$ CBL (2).

In a previous study we determined the configuration of both Igκ alleles in the 105 CBL (2). Per allele it was investigated whether the Igκ gene was germline, rearranged (Jκ rearrangement in the presence of the Cκ gene segment), or deleted (Jκ and/or Cκ deletion). Recently the same series of 105 leukemias was also analyzed for the configuration of the Igλ genes (Chapter 5, Table 1).

Half of the Igκ$^+$ CBL had one rearranged Igκ allele with the Igλ genes in germline configuration, while the other half had biallelic Igκ gene rearrangements or one rearranged and one deleted Igκ allele. Four Igκ$^+$ CBL also had Igλ gene rearrangements: one case in the group with biallelic Igκ rearrangements and three cases in the group with one rearranged and one deleted Igκ allele (Table 1). One of the latter three cases even had biallelic Igλ gene rearrangements. These data suggest that the IgL genes rearrange in a hierarchical order: Igκ rearrangement → Igκ deletion → Igλ rearrangement.

In the group of Igλ$^+$ CBL not a single case with biallelic Igκ rearrangements was observed. In fact, all cases had at least one deleted Igκ allele and ~75% of them had biallelic Igκ gene deletions. Approximately 25% of Igλ$^+$ CBL had biallelic Igλ gene

TABLE 1. IgL gene configuration of 53 Igκ⁺ and 52 Igλ⁺ chronic B-cell leukemias.

Igκ gene configuration	Igκ⁺ chronic B-cell leukemias Igλ gene configuration			Igλ⁺ chronic B-cell leukemias Igλ gene configuration		
	G/G	G/R	R/R	G/G	G/R	R/R
G/G	0	0	0	0	0	0
R/G	47% (25/53)	0	0	0	0	0
R/R	19% (10/53)	2% (1/53)	0	0	0	0
R/D	26% (14/53)	4% (2/53)	2% (1/53)	0	8% (4/52)	2% (1/52)
D/D	0	0	0	0	58% (30/52)	21% (11/52)
G/D	0	0	0	0	6% (3/52)	4% (2/52)

rearrangements; the majority (~75%) of these cases belonged to the group with biallelic Igκ gene deletions. These data support our conclusion that Igλ gene rearrangements are preceded by Igκ gene deletions, especially if it concerns biallelic Igλ gene rearrangements.

Our study provides information about both IgL alleles and distinguishes Igκ gene rearrangements from Igκ gene deletions. This resulted in a much more precise classification of the leukemias than in any previous study (Table 1). The combined data of the Igκ and Igλ genes in the two groups of CBL patients are not in line with the stochastic model of IgL gene rearrangements. According to the stochastic model, the Igκ/Igλ distribution of 1.4 in man should be accompanied with higher frequencies of Igλ gene rearrangements in Igκ⁺ B-cells, higher frequencies of Igκ gene rearrangements in Igλ⁺ B-cells, and essentially lower frequencies of Igκ gene deletions in Igλ⁺ B-cells.

The data in Table 1 illustrate that a hierarchical order of IgL gene rearrangement processes exists from germline (G), rearranged (R) to deleted (D): one Igκ allele rearranged (R/G) → further Igκ gene rearrangements (R/R) → one Igκ allele deleted (R/D) and occasionally one Igλ allele rearrangement (R/G) → both Igκ alleles deleted (D/D) and one or two Igλ gene rearrangements (G/R or R/R).

It has been suggested that the enhancer in the Jκ-Cκ intron plays a role in the ordered process of IgL gene rearrangements (11,12,16). However, this system is not full proof, because a few (10%) Igλ⁺ CBL cases had one germline Igκ allele in addition to a deleted Igκ allele. Based on the combined data, we conclude that the hierarchic model is much more dominant than the stochastic model in the regulation of human IgL gene rearrangement processes.

IgL gene rearrangement patterns and allelic exclusion

Until recently it was generally accepted that each lymphocyte expresses a single type of antigen receptor and that this single receptor expression is regulated via allelic exclusion (17). However, during the last few years, several reports indicated that dual receptor expression might occur in B-lymphocytes as well as in T-lymphocytes (18-21). It was found that a single T-lymphocyte might express two different T-cell receptor (TCR)β chains or two different TCRα chains, indicating that both TCRβ or both TCRα alleles are functionally rearranged *and* expressed (20, 21). Analogously, Giachino et al. demonstrated that in 0.2-0.5% of human B-lymphocytes dual expression of Igκ and Igλ occurs (18). This would imply that dual IgL chain expression is even higher, due to dual Igκ/Igκ and dual Igλ/Igλ expression. Although it will be difficult to prove the presence of dual Igκ/Igκ and dual Igλ/Igλ expression by immunophenotyping, the estimated dual IgL chain expression might be as high as 2%. Therefore the allelic exclusion mechanism probably is not strict, but shows some leakiness.

Table 1 shows that 8% (4 cases) of Igκ$^+$ CBL contained Igλ gene rearrangements and that 10% (5 cases) of Igλ$^+$ CBL contained Igκ gene rearrangements. Furthermore, 21% of Igκ$^+$ CBL had biallelic Igκ gene rearrangements and 27% of Igλ$^+$ CBL had biallelic Igλ gene rearrangements.

To get more insight in the allelic exclusion mechanisms in our series of CBL, we selected the four Igκ$^+$ CBL cases with Igλ gene rearrangements and the five Igλ$^+$ CBL cases with Igκ gene rearrangements for further study. Table 2 summarizes the configuration of the two Igκ alleles and the two Igλ alleles in these nine cases.

In our previous study on TCRγ and TCRδ genes in 13 TCRγδ$^+$ T-ALL, we found that biallelic complete TCRγ and TCRδ rearrangements occurred in ~85% and 45% of cases, respectively (22). Sequencing of the biallelic TCRγ and TCRδ junctional regions

TABLE 2. VL-JL gene configurations of the two groups of CBL that are framed in Table 1.

	Igκ genes		Igλ genes	
	allele 1	allele 2	allele 1	allele 2
Igκ$^+$ B-CLL with Igλ rearrangements				
- patient 1	R (Vκ-Jκ-Cκ)	R (Vκ-Jκ-Cκ)	R (J-Cλ3)	G
- patient 2	R (Vκ-Jκ-Cκ)	D (Vκ-Jκ-Kde)	R (J-Cλ1)	G
- patient 3	R (Vκ-Jκ-Cκ)	D (Vκ-Jκ-Kde)	R (J-Cλ2)	G
- patient 4	R (Vκ-Jκ-Cκ)	D (Vκ-Jκ-Kde)	R (J-Cλ2)	R (J-Cλ3)
Igλ$^+$ B-CLL with Igκ rearrangements				
- patient 5	R (Vκ-Jκ-Cκ)	D (Vκ-Kde)	R (J-Cλ2)	G
- patient 6	R (Vκ-Jκ-Cκ)	D (Vκ-Kde)	R (J-Cλ2)	G
- patient 7	R (Vκ-Jκ-Cκ)	D (Vκ-Jκ-Kde)	R (J-Cλ2)	G
- patient 8	R (Vκ-Jκ-Cκ)	D (Vκ-Kde)	R (J-Cλ3)	G
- patient 9	R (Vκ-Jκ-Cκ)	D (Vκ-Jκ-Kde)	R (J-Cλ3)	R (J-Cλ3)

Patient[a]	Vλ member[b]	Vλ		junctional region		Jλ	Jλ member	frame[c]
1.	not identified							
2.	IGLV3S2	GTAGTAGTGATCATCC	0	GGGG	-3	GTCTTCGGAACTGGG	Jλ1	+
3.	2e	GCAGGCACCTACACTT	0	GGGTG	-6	TTCGGCGGAGGG	Jλ2	+
4 a.	ψhslv2120	AGCAGTGCCACTT	-3	AAA	-2	GGTATTCGGCGGAGGG	Jλ2	-
b.	not identified							

Patient[a]	Vκ member[b]	Vκ		junctional region		Jκ	Jκ member	frame[c]
5.	A17	CAAGGTACAC	-10	TGACC	-4	CTTTTGGCCAG	Jκ2	-
6.	B3	CAATATTATAGTACTCC	-3	CCTG	0	TACACTTTTGGCCAG	Jκ2	+
7 a.	08/018	CAGTATGATAATCTCCC	-3	ATC	-2	CACTTTCGGCCCT	Jκ3	+
b.	B3	CAATATTATAGTACTCC	-3	CT	0	CTCACTTTCGGCGGA	Jκ4	-
8.	08/018	CAGTATGATAATCTCCCTC	-1	ACC	-6	TTCGGCGGA	Jκ4	-
9 a.	02/012	CAGAGTTACAGTAC	-6	GA	-9	GGCGGA	Jκ4	-
b.	not identified							

Figure 1. Junctional region sequences of Vλ-Jλ rearrangements in Igκ[+] CBL (patients 2, 3, 4) and Vκ-Jκ rearrangements in Igλ[+] CBL (patients 5, 6, 7, 8, 9). For each junctional region, the numbers of deleted nucleotides are indicated. The stop codon in the junctional region of patient 4 is underlined.

a. The VL-JL gene configurations of these patients are shown in Table 2.

b. The germline Vλ and Vκ sequences are from the "V-BASE GOLD" and/or published (IGLV3S2 and ψhslv2120) sequences (V Base Sequence Directory, Tomlinson et al., MRC centre for protein engineering, Cambridge, UK; or excession numbers L27696 and X71966).

c. In-frame: +; out-of-frame: -.

Patient 1: We were not able to amplify the Vλ-Jλ rearrangement.

Patient 4: A deletion of 36 nucleotides was found, starting from the last two codons of the Jλ2 gene segment up to 30 nucleotides in the J-Cλ intron, thereby deleting the Jλ splice site. We were not able to amplify the Vλ-Jλ rearrangement on the second allele.

Patient 5: This patient has a duplication of 55 nucleotides downstream in the VκII gene segment (A17), resulting in a stop codon.

Patient 7: RT-PCR analysis with Vκ and Cκ primers demonstrated that the in-frame rearrangement resides on the allele without deletion of the Cκ gene segment.

Patient 9: RT-PCR analysis with VκI(02/012)-Cκ primers was not successful, implying that the out-of-frame Vκ(02/012)-Jκ4 rearrangement resides on the allele with the Cκ deletion. The Vκ-Jκ rearrangement of the other allele could not be amplified.

revealed that in all cases one of the two alleles was in-frame, whereas the other allele was either out-of-frame or contained a stop codon in the junctional region (22). Therefore we assumed that sequence analysis of the junctional regions of the IgL genes in the nine analyzed CBL cases might explain the single IgL chain expression.

For sequence analysis, the VL-JL junctional regions were amplified by polymerase chain reaction (PCR), followed by heteroduplex analysis of the PCR products and subsequent sequencing. Sequencing of the VL-JL junctional regions started in frame work 1 (FR1) for the Vκ genes and in FR3 for Vλ genes down to the complete J gene segment. The preliminary results of our analyses are summarized in Figure 1. This figure shows that in at least two of the four studied Igκ+ CBL the Vλ-Jλ junctional region appeared to be in-frame. Additionally we searched for the occurrence of stop codons in the Vλ gene segments. Only in patient 4 we detected a stop codon in the Vλ-Jλ junctional region, which was also out-of-frame (Figure 1). We cannot exclude the existence of a stop codon upstream of the used FR3 primers. As compared to the known Vλ gene sequences, we found a few mismatches in the FR3 region of some Vλ's, but it should be noted that not all germline Vλ sequences are known. Furthermore, the mismatches in the FR3 region did not involve crucial amino acids, such as cysteins, which are important for folding of the protein chain.

Also in at least two of the Igλ+ CBL we found in-frame Vκ-Jκ junctional regions. One should realize that detection of in-frame Vκ-Jκ junctional regions in Igλ+ CBL does not necessarily imply that it concerns a functional rearrangement, because this in-frame rearrangement might reside on an allele with deletion of the Cκ gene segment (see patients 7 and 9 in Table 2). This can be further investigated at the RNA level by use of RT-PCR analysis with Vκ-Cκ primers. Using this approach we demonstrated that the in-frame Vκ 08/018-Jκ3 rearrangement of patient 7 was transcribed into complete Vκ-Cκ mRNA (Figure 1).

Our preliminary results indicate that several Igκ+ CBL and Igλ+ CBL have in-frame Igλ genes and in-frame Igκ genes, respectively, which might be functional. Therefore dual IgL chain expression might occur in CBL, if no allelic exclusion mechanisms are active. However, immunophenotyping of ~300 CBL cases in our diagnostic laboratory during the last decade did not reveal a single case with dual Igκ/Igλ expression. This implies that the frequency of dual IgL chain expression in B-cell malignancies might be comparable or lower than the 0.2-0.5% in normal B-cells and that allelic exclusion might be regulated at the transcription, translation, or post-translation level (18).

CONCLUSION

The development of the new set of Igλ probes has further improved the possibilities for diagnostic clonality studies in suspect B-cell proliferations. These probes do not only allow *detection* of clonal Igλ gene rearrangements, but also the *identification* of these rearrangements. Furthermore, the new tools allowed us to perform detailed analysis of the configuration of IgL genes in B-cell malignancies and thereby to study the stepwise IgL gene rearrangement processes. These studies demonstrated that the hierarchical

model is much more dominant than the stochastic model in the regulation of IgL gene rearrangements.

Additional IgL gene studies provided insight into the mechanism of allelic exclusion. In most B-cells probably only one Igκ or Igλ allele is functionally rearranged, but in ~4% of the studied B-cell malignancies an Igκ *and* Igλ allele seemed to be functional, although only a single IgL chain was expressed on the cell surface. This implies that allelic exclusion mechanisms might act at the transcription, translation, or post-translation level. These allelic exclusion studies in lymphoid malignancies can be further extended to B-cell malignancies with biallelic Igκ gene rearrangements or biallelic Igλ gene rearrangements and to T-cell malignancies with biallelic TCR gene rearrangements. Such studies are important for understanding of the development of the Ig/TCR gene repertoire and the Ig/TCR receptor editing.

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SUMMARY

The specific immune system consists of B- and T-lymphocytes with antigen specific receptors on their cell surface. Each lymphocyte has one type of antigen receptor, allowing the recognition of one particular antigen. B-lymphocytes recognize antigens by membrane bound immunoglobulin (Ig) molecules, also called B-cell receptors (BCR). The antigen-specific receptors of T-lymphocytes are called T-cell receptors (TCR). Ig molecules are heterodimers which consist of two Ig heavy (IgH) chains, joined by disulfide bonds, and two Ig light (IgL) chains, each of which is linked to one of the IgH chains. Two types of IgL chains exist: Ig kappa (Igκ) and Ig lambda (Igλ). Each Ig chain consists of a variable (V) domain and one or more constant (C) domains. The variable domains are encoded in the DNA by exons, which consist of V, D, and J gene segments in case of IgH chains and V and J gene segments in case of Igκ and Igλ chains. These gene segments are joined via rearrangement processes during early B-cell differentiation.

B-cells originate in the bone marrow (BM) from lymphoid progenitor cells, which differentiate via precursor-B-cells to B-lymphocytes and finally to Ig/antibody secreting plasma cells. During this differentiation pathway B-cells undergo several gene rearrangements, such as V-D-J recombination of IgH chain genes and V-J recombination of IgL chain genes, secondary rearrangements (V replacements, V-J replacements, etc.) during receptor editing, and IgH class switch rearrangements. Additionally somatic hypermutation occurs which can improve the affinity of the Ig molecules.

Virtually all malignancies are derived from a single malignantly transformed cell. Therefore all cells of a B-cell malignancy have identically rearranged Ig genes. These clonal Ig gene rearrangements can be detected by Southern blot analysis. Until recently, optimal DNA probes were only available for IgH and Igκ genes, but not for Igλ genes, although Igλ gene rearrangements are found in ~40% of B-cell malignancies. Therefore it would be convenient to have appropriate DNA probes for Igλ gene analysis as well.

The aim of this thesis was twofold: firstly, to develop tools to detect and to identify clonal Igλ gene rearrangements in B-cell malignancies and to analyze the Igλ gene rearrangement patterns in the various types of B-cells malignancies (Chapters 3-6). Secondly, to investigate the occurrence of ordered or stochastic rearrangement patterns of IgL genes and to investigate the allelic exclusion mechanisms of IgL genes, i.e. the phenomenon that a B-cell expresses a single type of IgL chain (either κ or λ) on the cell surface membrane (Chapter 7).

Chapter 2 deals with the technical aspects of Southern blot analysis of Ig genes in B-cell malignancies. The protocols as well as pitfalls for several steps of Southern blot analysis are discussed. To be able to detect the Igλ gene rearrangements by Southern blot analysis for diagnostic purposes, we firstly determined the precise restriction map of the Igλ gene, designed a general Igλ DNA probe (IGLC3), and carefully selected the appropriate restriction enzymes (Chapter 3). Our Southern blot analyses of 75 Igλ$^+$

B-cell malignancies with the IGLC3 probe in *Eco*RI/*Hin*dIII digests allowed the detection of clonal Igλ gene rearrangements in 95% of the patients and in 94% of the 98 rearranged Igλ alleles (Chapter 3). In contrast, *Hin*dIII and *Eco*RI single digests allowed detection of rearrangements in only 78% and 83% of the patients, and 67% and 79% of the rearranged alleles, respectively. Therefore, we conclude that usage of the IGLC3 probe in combination with *Eco*RI/*Hin*dIII is optimal for the detection of Igλ gene rearrangements in Igλ$^+$ B-cell malignancies.

Subsequently, we wished to identify the rearrangements within the Igλ locus. Therefore we developed a set of seven 'Igλ isotype specific' DNA probes: the IGLC1D probe for the J-Cλ1 gene region, the IGLC2D probe for the J-Cλ2 gene region, the IGLJ2 probe for the highly homologous J-Cλ2 and J-Cλ3 gene regions, and the IGLC4D, IGLJ5, IGLJ6, and IGLJ7 probes for the last four J-Cλ gene regions, respectively. We carefully selected appropriate restriction enzymes (*Hin*dIII, *Bgl*II, *Bam*HI, and/or *Eco*RI) for each of the seven Igλ isotype specific DNA probes (Chapter 4). These seven probes indeed allowed detection and indentification of all Igλ gene rearrangements. Subsequently, these Igλ isotype specific DNA probes were used for analysis of a large series of 212 B-cell malignancies to determine the Igλ isotype rearrangement patterns (Chapter 5). This study on 76 precursor-B-cell acute lymphoblastic leukemias (ALL), 74 Igλ$^+$ chronic B-cell leukemias (CBL), 34 Igλ$^+$ B-cell non-Hodgkin lymphomas (B-NHL), and 28 Igλ$^+$ multiple myelomas showed that most rearrangements occurred to the J-Cλ3 gene region (~50%), followed by J-Cλ2 rearrangements (30-40%) and J-Cλ1 rearrangements (~10%). Rearrangements to the J-Cλ6 and J-Cλ7 gene regions were rare, while no rearrangements occurred to J-Cλ4 and J-Cλ5. The absence of J-Cλ4 and J-Cλ5 rearrangements is probably due to the inappropriate recombination signal sequences of the Jλ4 and Jλ5 gene segments. The few J-Cλ6 gene rearrangements were only found in precursor-B-ALL, not in Igλ$^+$ B-cell malignancies. This may be due to the fact that the J-Cλ6 gene region can only encode for a truncated Igλ chain, and not for a complete Igλ chain. A J-Cλ7 rearrangement was found only once. This is in line with the recent report by Nieuwold et al. (1996), who demonstrated that the J-Cλ7 region encodes for the newly identified Mcp Igλ isotype, which is rarely expressed (<1%).

Our study also shows that preferential Igλ isotype usage might depend on the maturation stage of the B-cell malignancies: J-Cλ2 rearrangements were detected in 32% of CBL and B-NHL cases, while J-Cλ3 rearrangements occurred in 54% of these cases. However, in the fully differentiated multiple myelomas J-Cλ2 gene rearrangements occurred in 60%, while only 37% of the rearrangements occurred in the J-Cλ3 gene region. This shift from preferential J-Cλ3 rearrangements in CBL and B-NHL to preferential J-Cλ2 rearrangements in multiple myelomas might be due to selection preceding the malignant transformation.

Our study on 212 B-cell malignancies also reveals that 97% of all Igλ gene rearrangements occurred to J-Cλ1, J-Cλ2, or J-Cλ3 gene regions. Based on this information, we evaluated whether the extensive Southern blot analysis of all seven Igλ regions (20 hybridizations) can be restricted to the application of J-Cλ1, J-Cλ2, and J-Cλ3 specific probes (the IGLC1D, IGLC2D, and IGLJ2 probes) and a few restriction

enzyme digests. We found that the three probes in combination with *Bgl*II and *Eco*RI/*Hin*dIII digestion (5 hybridizations) allow detection and identification of 96% of all J-Cλ gene rearrangements in all B-cell malignancies (Chapter 6).

The second part of this thesis concerns the investigation of ordered or stochastic rearrangement patterns of IgL genes and the investigation of allelic exclusion mechanisms of IgL genes. The precise rearrangement patterns of both Igκ alleles and both Igλ alleles were determined in 53 Igκ$^+$ CBL and 52 Igλ$^+$ CBL. The results clearly demonstrated that IgL gene rearrangements occur in an hierarchical order with Igκ gene rearrangements, followed by Igκ gene deletions, whereafter the Igλ genes rearrange (Chapter 7). Nevertheless four Igκ$^+$ CBL also contained Igλ gene rearrangements and five Igλ$^+$ CBL also contained Igκ gene rearrangements. These nine cases were selected for further analysis. We were able to sequence several VL-JL junctional regions to determine the status of the rearranged Igλ alleles in Igκ$^+$ CBL and the rearranged Igκ alleles in the Igλ$^+$ CBL (Chapter 7). The literature on allelic exclusion supposes that non-expressed, rearranged IgL alleles are out-of-frame and/or have a stop codon and therefore cannot encode for an IgL chain. However, the preliminary results of our sequence analyses demonstrate that the non-expressed alleles can be in-frame. This implies that allelic exclusion of Igλ genes in these cases is probably regulated at the transcriptional, translational, or posttranslational level.

In conclusion, this thesis describes the development of tools for the detection and identification of clonal Igλ gene rearrangements and the application of these tools for analysis of Igλ gene rearrangement patterns in B-cell malignancies. Secondly, we demonstrated that the IgL gene rearrangements occur in an hierarchial order and that allelic exclusion of IgL genes may also be regulated at the transcriptional or (post)translational level.

SAMENVATTING

Het specifieke immuunsysteem bestaat uit B- en T-lymfocyten met antigeenspecifieke receptoren op de celmembraan. Elke lymfocyt heeft één type antigeenreceptor, waarmee één bepaald antigeen kan worden herkend. B-lymfocyten herkennen antigenen via hun membraangebonden immunoglobuline (Ig) moleculen, ook wel B-celreceptoren genoemd. De antigeenspecifieke receptoren van T-lymfocyten worden T-celreceptoren (TCR) genoemd. Ig moleculen zijn heterodimeren die bestaan uit twee zware en twee lichte ketens, met elkaar verbonden via zwavelbruggen. Aan iedere zware keten is één lichte keten gebonden. Er bestaan twee soorten Ig lichte ketens: Ig kappa (Igκ) en Ig lambda (Igλ). Iedere Ig keten bestaat uit één variabele en één of meer constante domeinen. De variabele domeinen worden in het DNA gecodeerd door exonen, die in het geval van Ig zware ketens bestaan uit V, D en J gensegmenten en in het geval van Ig lichte ketens uit V en J gensegmenten. Deze gensegmenten worden tijdens de vroege B-celdifferentiatie aan elkaar gekoppeld door een herschikkingsproces.

B-cellen worden in het beenmerg gevormd uit lymfoide voorlopercellen, die verder uitrijpen tot voorloper-B-cellen, vervolgens tot rijpe B-cellen en tenslotte tot plasmacellen die Ig/antistoffen secerneren. Gedurende de differentiatie ondergaan de B-cellen een aantal malen genherschikkingen, zoals V-D-J herschikkingen van de Ig zware ketengenen en V-J herschikkingen van de Ig lichte ketengenen, secundaire herschikkingen (V replacements, V-J replacements, etc.) gedurende de zogenaamde 'receptor editing' en tenslotte de Ig zware keten klasseswitch. Tevens treedt somatische hypermutatie op, wat kan leiden tot verhoogde affiniteit van de antigeenreceptoren van de B cellen.

Bijna alle maligniteiten ontstaan uit één enkele maligne ontaarde cel. Daardoor hebben alle cellen van een B-cel maligniteit hun Ig genen exact op dezelfde wijze herschikt. Deze klonale Ig genherschikkingen kunnen door middel van Southern blotanalyse gedetecteerd worden. Tot voor kort waren alleen DNA probes beschikbaar voor de analyse van Ig zware ketengenen en Igκ genen, maar niet voor Igλ genen. Omdat ~40% van de B-cel maligniteiten van het Igλ isotype zijn, was het belangrijk om geschikte DNA probes te ontwikkelen voor de analyse van deze maligniteiten op Igλ gen-niveau.

Het doel van dit proefschrift was tweeledig. Ten eerste het ontwikkelen van technieken waarmee klonale Igλ genherschikkingen in B-cel maligniteiten kunnen worden geïdentificeerd, zodat de Igλ genherschikkingspatronen in de verschillende B-cel maligniteiten kunnen worden geanalyseerd (Hoofdstukken 3-6). Ten tweede, het onderzoeken of de herschikking van de Ig lichte ketengenen geordend of stochastisch plaatsvindt, en het analyseren van het mechanisme van allelische exclusie van de Ig lichte ketengenen (Hoofdstuk 7). De allelische exclusie zorgt ervoor dat op een B-cel slechts één enkel type Ig lichte keten (κ of λ) tot expressie komt.

Hoofdstuk 2 behandelt de technische aspecten van Southern blotanalyse van de Ig genen in B-cel maligniteiten. Methoden en problemen bij de verschillende stappen worden bediscussieerd. Om Igλ genherschikkingen door middel van Southern blotanalyse

te kunnen detecteren voor diagnostische doeleinden, is allereerst de precieze restrictiekaart van het Igλ gen bepaald en een algemene Igλ DNA probe (IGLC3) ontworpen. Vervolgens werden geschikte restrictie enzymen geselecteerd (Hoofdstuk 3). Analyse op basis van de combinatie van de IGLC3 probe en *Eco*RI/*Hin*dIII digesten van 75 Igλ$^+$ B-cel maligniteiten resulteerde in de detectie van Igλ genherschikkingen bij 95% van de patiënten en 94% van de 98 herschikte Igλ allelen. In digesten met *Hin*dIII óf *Eco*RI werd bij respectievelijk 78% en 83% van de patiënten en bij 67% en 79% van de herschikte allelen een herschikking gedetecteerd. Wij concluderen hieruit dat het gebruik van de IGLC3 probe in combinatie met *Eco*RI/*Hin*dIII digestie optimaal is voor de detectie van Igλ genherschikkingen in Igλ$^+$ B-cel maligniteiten.

Vervolgens wilden wij de herschikkingen binnen het Igλ locus kunnen indentificeren. Daartoe hebben we zeven nieuwe Igλ 'isotype specifieke' DNA probes ontwikkeld: IGLC1D voor de J-Cλ1 gen regio, IGLC2D specifiek voor de J-Cλ2 genregio, IGLJ2 voor de sterk homologe J-Cλ2 en J-Cλ3 genregionen samen, en IGLC4D, IGLJ5, IGLJ6 en IGLJ7 voor de resterende vier genregionen. Passende restrictie enzymen werden geselecteerd (*Hin*dIII, *Bgl*II, *Bam*HI, en/of *Eco*RI) voor elk van de zeven Igλ isotype specifieke DNA probes (Hoofdstuk 4). Deze Igλ isotype specifieke DNA probes werden gebruikt om Igλ isotype genherschikkingspatronen te analyseren in 212 B-cel maligniteiten, bestaande uit 76 voorloper-B-cel acute lymfoblastaire leukemieën (ALL), 74 Igλ$^+$ chronische B-cel leukemieën (CBL), 34 Igλ$^+$ B-cel non-Hodgkin lymfomen (B-NHL), en 28 Igλ$^+$ multiple myelomen (Hoofdstuk 5). Deze studie liet zien dat de meeste herschikkingen in het J-Cλ3 gebied hadden plaatsgevonden (~50%), gevolgd door J-Cλ2 herschikkingen (30-40%), en J-Cλ1 herschikkingen (~10%). Herschikkingen naar J-Cλ6 en J-Cλ7 bleken in deze serie zeldzaam, terwijl geen herschikking werd gevonden naar J-Cλ4 en J-Cλ5 genregionen. De afwezigheid van deze herschikkingen wordt waarschijnlijk veroorzaakt door een ongeschikte recombinatie signaal sequentie van Jλ4 en Jλ5 gensegmenten. De J-Cλ6 genherschikkingen werden alleen gevonden in voorloper-B-ALL en niet in Igλ$^+$ B-cel maligniteiten. Een mogelijke verklaring hiervoor is dat J-Cλ6 niet voor een complete Igλ keten codeert. Een J-Cλ7 genherschikking werd slechts eenmaal gevonden in deze serie. Dit komt overeen met het recentelijk verschenen artikel van Niewold et al. (1996), die aantoonden dat J-Cλ7 codeert voor een niet eerder gedefinieerd Igλ isotype, Mcp, dat slechts zelden tot expressie komt (<1%).

Onze studie laat tevens zien dat het preferentiële Igλ isotype gebruik afhangt van het rijpingsstadium van de B-cel maligniteit: J-Cλ2 herschikkingen werden gedetecteerd in 32% van de CBL en B-NHL, terwijl J-Cλ3 herschikkingen in 54% van de CBL en B-NHL werden gevonden. In de geheel uitgedifferentiëerde multiple myelomen vond 60% van de herschikkingen in het J-Cλ2 gebied plaats en slechts 37% in J-Cλ3. Deze verschuiving van een voorkeur voor J-Cλ3 in CBL en B-NHL naar J-Cλ2 in multiple myelomen wordt waarschijnlijk veroorzaakt door selectie voorafgaand aan de maligne transformatie.

Uit onze studie is gebleken dat 97% van alle Igλ genherschikkingen de J-Cλ1, J-Cλ2 of J-Cλ3 regio betroffen. Op grond van deze informatie hebben wij onderzocht of de uitgebreide Southern blotanalyse van alle Igλ genregionen (ongeveer 20 hybridizatie-

stappen) beperkt kan worden tot de analyse van de meest gebruikte gengebieden met bijbehorende DNA probes (IGLC1D, IGLC2D en IGLJ2) in combinatie met enkele digesten. Wij vonden dat deze drie probes in combinatie met *Bgl*II en *Eco*RI/*Hin*dIII digesten (5 hybridizatiestappen) resulteerden in de detectie en identificatie van 96% van alle J-Cλ herschikte genen in alle B-cel maligniteiten (Hoofdstuk 6).

Het tweede deel van dit proefschrift betreft onderzoek naar de geordende of stochastische herschikkingspatronen van de genen die coderen voor de Ig lichte keten en het onderzoek naar allelische exclusiemechanismen gedurende de Ig lichte keten genherschikkingen. De preciese herschikkingspatronen van zowel Igκ als Igλ allelen werden bepaald in 53 $Ig\kappa^+$ CBL en 52 $Ig\lambda^+$ CBL. De analyse hiervan liet duidelijk zien dat Ig lichte keten genherschikkingen in een hiërarchische volgorde plaatsvinden; Igκ genherschikkingen treden als eerste op, gevolgd door Igκ gendeleties, waarna de Igλ genen beginnen met herschikken (Hoofdstuk 7). Desalniettemin vonden wij vier $Ig\kappa^+$ CBL die ook Igλ genherschikkingen hadden en vijf $Ig\lambda^+$ CBL die Igκ genherschikkingen hadden. Deze negen gevallen werden verder onderzocht. De 'junctional regions' van verschillende V-J lichte keten genherschikkingen zijn geanalyseerd voor hun nucleotidensequentie, om de 'reading frame' status te bepalen van de herschikte Igλ allelen in de $Ig\kappa^+$ CBL, en van de herschikte Igκ allelen in de $Ig\lambda^+$ CBL. Een algemene aanname in de literatuur is dat het herschikte Ig lichte keten allel, dat niet tot expressie komt, out-of-frame is en/of een stop codon bevat en daardoor niet tot een functionele Ig lichte keten kan leiden. Daarentegen laten onze voorlopige resultaten zien dat het niet-geëxpresseerde allel toch 'in-frame' kan zijn. Dit impliceert dat allelische exclusie ook gereguleerd zou worden op het niveau van transcriptie, translatie of zelfs post-translatie.

Samenvattend, beschrijft dit proefschrift de ontwikkeling van methoden om klonale Igλ genherschikkingen te detecteren en te identificeren en de toepassing ervan voor de bepaling van Igλ genherschikkingspatronen in B-cel maligniteiten. Voorts hebben wij aangetoond dat de Ig lichte keten genherschikkingen in een hiërarchische volgorde plaatsvinden en dat allelische exclusie van de Ig lichte ketengenen waarschijnlijk ook op het niveau van transcriptie en (post)translatie kan worden gereguleerd.

ABBREVIATIONS

ALL	:	acute lymphoblastic leukemia
B-ALL	:	B-cell acute lymphoblastic leukemia
B-CLL	:	B-cell chronic lymphocytic leukemia
BCR	:	B-cell receptor
BM	:	bone marrow
B-NHL	:	B-cell non-Hodgkin lymphoma
B-PLL	:	B-cell prolymphocytic leukemia
CBL	:	chronic B-cell leukemia
C	:	constant
CDR	:	complementarity determining region
CLL	:	chronic lymphocytic leukemia
Cy	:	cytoplasmic expression of proteins (e.g. CyIg, CyCD79)
D	:	diversity
enh	:	enhancer
FCL	:	follicular cell lymphoma
FR	:	frame work
HCL	:	hairy cell leukemia
HCLv	:	HCL variant
HVP	:	hypervariable polymorphic region
Ig	:	immunoglobulin
IgH	:	Ig heavy
Igκ	:	Ig kappa
IgL	:	Ig light
Igλ	:	Ig lambda
IL-2	:	interleukin-2
J	:	joining
kb	:	kilobase
kDa	:	kilodalton
Kde	:	kappa deleting element
McAb	:	monoclonal antibody
MCL	:	mantle cell lymphoma
MM	:	multiple myeloma
MNC	:	mononuclear cells
NHL	:	non-Hodgkin lymphoma
PB	:	peripheral blood
PCR	:	polymerase chain reaction
pre-B-ALL	:	precursor-B-ALL
pre-BCR	:	pre-B-cell receptor
RAG	:	recombination activating gene

RFLP	:	restriction fragment length polymorphism
RSS	:	recombination signal sequences
SLVL	:	splenic lymphoma with villous lymphocytes
SmIg	:	surface membrane Ig (molecules)
TCR	:	T-cell receptor
TdT	:	terminal deoxynucleotidyl transferase
T-NHL	:	T-cell non-Hodgkin lymphoma
V	:	variable
ψ	:	pseudo
ψLC	:	pseudo light chain

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Grote gedachten komen uit het hart

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