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REVIEW ARTICLE

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Gene rearrangements and chromosomal translocations in T cell lymphoma – diagnostic applications and their limits

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Abstract The diversity of the T cell receptor (TCR) repertoire is established for individual T lymphocytes by developmentally regulated gene rearrangements and shaped by predominantly intrathymic selection procedures. TCR gene probes in Southern blot experiments and TCR primers for the polymerase chain reaction (PCR) help to distinguish polyclonal from abnormal clonal T cell proliferations and to monitor clonal disease after treatment. Rearrangement studies can identify the lineage and developmental stage of a lymphocyte clone. Cross-lineage rearrangements, false positive or negative results are rarely misleading when morphology and immunophenotypical findings are considered. Rearrangement studies, however, have not contributed significantly to the comprehension of lymphomagenesis. Analyses of characteristic chromosomal translocations in T cell leukaemias and lymphomas may provide further insight into the mechanisms of malignant transformation. Transcription factors are often involved and sometimes abnormally transcribed, which may alter the physiological intracellular signalling in T cells. Interphase cytogenetic analysis by chromosomal fluorescence in situ hybridization (FISH) has become a new tool in the search for transformed T cells carrying specific translocations. Archival biopsy material is now accessible for PCR rearrangement studies and FISH cytogenetics. This adds another dimension to the diagnosis, disease monitoring and biological understanding of malignant T cell lymphomas and leukaemias.

Key words Rearrangement · T cell receptor · Polymerase chain reaction · Translocation · Fluorescence in situ hybridization · T cell lymphoma

Introduction

The classification of T cell lymphomas is difficult. Malignant transformed T lymphocytes are often mixed with abundant reactive haematopoietic cells. Small T cell clones may remain morphologically unrecognized among inflammatory lymphocytes, epithelioid cells, macrophages, eosinophilic granulocytes and immune accessory cells. The variegated histological picture in most low-grade T cell lymphomas differs from usual low-grade B cell lymphomas that present with a monomorphic or clearly compartmentalized infiltration pattern. Diagnosis of peripheral T cell lymphomas very often requires immunohistochemical investigations besides a careful and knowledgeable cytomorphological analysis. Monoclonality, a reliable indicator of an abnormal lymphoproliferation, is not detectable by immunophenotyping in T cell tumours. It is now possible to recognize clonality following cloning of the human T cell receptor (TCR) β chain using molecular methods [124, 183]. Three additional TCR chain genes have also been characterized (TCRα, TCRγ and TCRδ) and this has not only helped in the diagnosis of T cell neoplasms but has also increased our understanding of T cell ontogeny and differentiation. DNA analysis is now an additional tool for the diagnosis and monitoring of T cell malignancies and although of value in the diagnosis of lymphoproliferative disease it has its limitations. In recent years, nonrandom chromosomal abnormalities have been identified in T cell neoplasms with increasing frequency, opening a new diagnostic avenue. Probes for non-radioactive chromosomal in situ hybridization (ISH) and primers for polymerase chain reaction (PCR)-based detection are obtained by molecular cloning and sequencing of the breakpoint flanking regions.

Current knowledge about molecular genetical rearrangements and their usefulness and limits in the diagnosis of malignant T cell lymphomas is the focus of this review. Non-random chromosomal rearrangements will be discussed which involve TCR gene loci or may contribute to the malignant transformation of T lymphocytes.

The T cell receptor

T lymphocytes are generated from lymphopoietic precursor cells in the thymus. After survival of a radical in-

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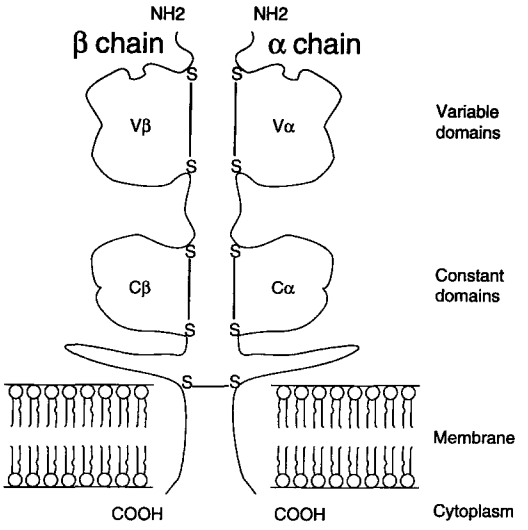

Fig. 1 Schematic drawing of the membrane-bound αβ T cell receptor (TCR) depicting its extracellular variable (*V*) domains, the invariable extracellular and the transmembrane domain, and the short cytoplasmic portion of the constant (*C*) region. The two TCR chains are covalently bound by sulfhydral groups on their cystein residues

trathymic selection process they carry antigen-recognition molecules, the TCRs, on the cell surface. These disulphide-linked heterodimeric polypeptide molecules consist mainly of α/β chains (Fig. 1). In 5–10% of mature T lymphocytes an alternative TCR is found composed of γ/δ chains [20]. The TCR chains attain a large diversity by the random rearrangement of multiple variable (V), diversity (D; in TCRβ or δ genes) and short joining (J) genes which together encode the extracellular variable domain. Constant regions at the carboxy terminus encode an invariable extracellular, a transmembrane and a short intracellular domain (Fig. 1). Mature T cells specifically recognize antigens from outside or neoantigens from within the host organism with their TCR V domains. These commonly interact with antigens only when bound to special presentation molecules encoded by the major histocompatibility complex (MHC) [191]. Specific antigen binding transforms T lymphocytes into blast cells and initiates their clonal expansion.

Generation of V region diversity of the T cell receptors

The individual V region assembly generates an estimated 10^{19} different combinations for the TCR αβ molecules [40]. The combinatorial diversity of the TCRγδ molecules is lower [48] because of the limited number of functional Vγ/δ, Jγ/δ, and Dδ segments that can rearrange and form functional TCRγ and δ chain genes [140]. However, actual numbers may differ because the repertoire can be greatly shaped by genomic deletions of long stretches of V gene segments [9, 93, 129]. Positive or negative selection of T cells expressing certain TCR VDJ combinations during thymic maturation or post-thymic circulation, and the momentary activation of lymphocyte subsets by environmental antigens such as bacterial or retroviral superantigens may also alter the TCR V region repertoire [16, 84, 85, 149].

A set of conserved nucleotides, heptamers and nonamers flanks the germline V, D, and J segments. They function as recombination signal sequences for V-D, D-J, or V-J joining, and are recognized by a recombinase enzyme system [184]. These signal sequences are separated by a nonconserved spacer. The spacer situated 3′ of the V or D gene segment is 21–23 base pairs (bp) long (about two turns of the DNA helix). It is 11–12 bp long (about one turn of the double helix) when located 5′ of the D or J gene segment. Flanking sequences with a one-turn spacer signal can only rearrange to a two-turn signal, ensuring joining of appropriate gene segments. Thus, one V and one J can recombine, but more than one D segment can join. This has frequently been described for Dδ1-Dδ2, Dδ1-Dδ3 and Dδ1-Dδ2-Dδ3 joinings [13]. Most commonly, the coding joint stays in the chromosome and a circular DNA molecule containing the signal joint and intervening sequences is excised [132]. Intervening DNA stretches are retained, if the two segments are joined in an opposite transcriptional orientation (conversion) [117]. At the coding ends, the joining is commonly imprecise. This happens through differential trimming of recombining gene termini by exonucleases and through duplication of one or two nucleotides at the recombination cleavage sites (P-nucleotides) [99]. Introduction of up to 15 nucleotides between V-D, D-D, D-J or V-J junctions in every possible random sequence generates non-template (N) diversity. The enzyme terminal desoxynucleotidyl transferase (TdT) probably mediates addition of these N-nucleotides [102]. N diversity contributes most significantly to the variability of the immune receptors, but it may also result in the generation of stop codons at the coding junctions [2]. Somatic hypermutation, one major mechanism for immunoglobulin (Ig) V region diversity, seems not to occur in TCR genes [40, 140].

The recombinase activity is at least in part initiated by products of two recombinase activating genes, RAG-1 and RAG-2. Expression of these genes correlates strictly with V(D)J recombinase activity. Their transcripts occur in pre-B and pre-T cells but not in later stages of lymphocyte development [148]. During T cell development, RAG-1 gene expression does not seem to occur in prothymocytes or mature thymocytes either [186]. It is expressed in two waves, in double negative (DN) CD4-CD8- and double positive (DP) CD4+CD8+ thymocytes, reflecting the start of TCRβ rearrangement and the transcription of the TCRα chain, respectively [49]. Vβ and Jβ heptamers are virtually indistinguishable from those found next to Ig genes suggesting that the recognition devices for Ig and TCR gene rearrangements are very similar. This may be the reason why T cells occasionally have D-J rearrangements of the Ig heavy chain (IgH) genes [97].

Genomic organization of the T cell receptors

The four TCR polypeptides generally form mutually exclusive pairs of TCRαβ or TCRγδ heterodimers [40]. The different molecules have a very similar but not identical gene structure (Fig. 2).

TCR α chain genes map to the long arm of chromosome 14 (14q11–12) [25] and harbours the TCR δ genes between its V and J region gene sequences. Approximately 50 V α genes [188] are spread out over more than 750 kb [62]. More than 80 Jα gene segments are dispersed over an area of at least 50 kb of DNA [8, 28, 13, 165]. D region coding sequences have not been identified but N region diversification exists in the V-J junctional regions [91]. The α chain locus contains only one C region gene composed of four exons [13].

TCR δ chain gene sequences occupy the space between the Vα and Jα gene cluster. Only one Cδ region gene exists. Three joining gene segments (Jδ 1–3) localize 3.4, 5.7, and 12 kb upstream of the first Cδ exon [159]. Most early fetal δ rearrangements involve C-proximal Jδ segments (Jδ3) while most δ chains at a later developmental stage in peripheral T cells use C-distal Jδ segments (Jδ1). Until now, ten different Vδ genes have been characterized with six Vδ genes being functional. Vδ3 maps 3′ to Cδ and upstream to Jα genes in an inverse orientation. Most of the γδ T cells use Vδ1 or Vδ2 [160], and T cells or early ontogenic origin use predominantly Vδ2 segments [95]. V region segments can be shared between the TCR α and δ locus [20, 67]. Four D gene segments have been characterized [41, 112]. Since joining of several D segments can occur during recombination, variability of γ/δ heterodimers might be considerably higher than one would expect from their limited number of functional V genes [13]. α/β TCR bearing cells usually delete both alleles of the δ locus.

The TCR β chain locus spans about 600 kb on chromosome 7q35 [24]. More than 60 different V region genes belong to 20 subfamilies [89]. They are mapped to the 5′ end of the two D-J-C clusters [100]. All Vβ genes rearrange to both Jβ clusters with similar frequency [40, 190]. The two constant region genes of the β chain are approximately 8 kb apart from each other. Their amino acid sequences are highly homologous [166]. Two J gene clusters, Jβ1 and Jβ2, are each located 2–5 kb 5′ to the constant region genes, containing six and seven J gene segments, respectively. Rearrangements in the TCR β locus more frequently involve D-Jβ2 than D-Jβ1 joinings which results in the deletion of the DJCβ1 gene segments [186].

The TCR β chain gene maps to the short arm of chromosome 7 (7p15) where it spans a distance of approximately 160 kb [157]. Only 10 of the 15 known Vγ segments undergo rearrangement, and only 8 are functional (V2–5, V8–11) [33, 55]. The other V genes are pseudogenes that are not expressed at the protein level due to transcriptional or translational defects. The TCRγ locus contains five known joining segments, divided in two groups comprising Jγ1.1, Jγ1.2, and Jγ1.3 located upstream of Cγ1, and Jγ2.1 and Jγ2.3 located upstream of Cγ2 [138]. Both Cγ segments are structurally similar to the Cα and Cβ genes, but have three instead of four exons [106]. Exon II sequences are different between Cγ1 and Cγ2. Cγ2 has two or three copies of the second exon but none of these code for the cysteine residue thought to be important for interchain disulfide linkage [94]. Thus, it depends on the Cγ gene usage whether (Cγ1) or not (Cγ2) the TCR γ chain can form a disulfide bond with the TCR δ chain [76].

The exact order in which the different TCR chain genes rearrange is unknown. Studies in the murine system and molecular analyses of thymic T cell leukaemias

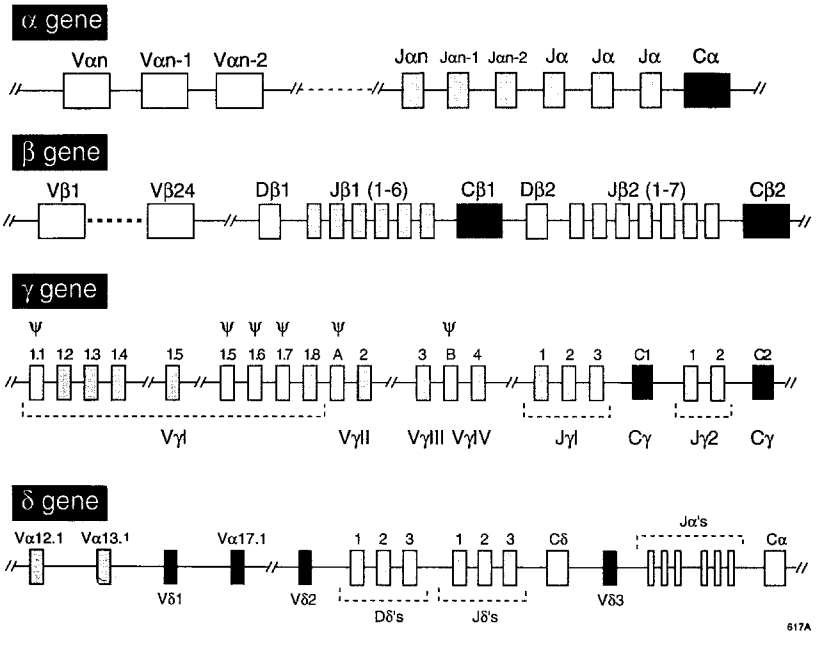

Fig. 2 Organization of the human TCR genes. All four TCR gene loci contain multiple *V* region genes and several joining (*J*) segments 5′ to either one (TCRα or δ) or two (TCRβ or γ) *C* genes. Diversity (*D*) genes have been identified for the TCRβ and δ chains. The TCRγ locus contains multiple V pseudogenes (ψ). The TCRδ genes are nested between the *Vα* and *Jα* gene clusters. The *Vδ3* gene segment maps 3′ to the *Cδ* locus in an inverse orientation

provide some insight in the step-wise recombination processes. Rearrangements in the TCR δ locus appear to precede recombination of the other TCR genes [17, 43, 107]. The TCR γ genes start to rearrange shortly before the TCR β genes. Productive TCR β rearrangement on one allele leads to receptor protein translation. This blocks recombination of the TCR β locus on the other chromosome, a phenomenon termed allelic exclusion [170].

Rearrangement at the TCRβ locus occurs at the DN stage and precedes rearrangement at the TCRα locus [27, 53, 141]. Unlike the TCRβ protein, the TCRα protein does not prevent further TCRα rearrangements. Thus, mature T cells can have two productive TCRα genes and may express two TCRs with different α chains [134]. After expression of functional TCRs and during positive and negative selection only few thymocytes differentiate into mature single positive cells [153].

CD4-8-αβT cells form about 0.5% of peripheral T cells and may use antigen-presenting cells, restriction molecules, and selection routes different from those used by antigen-specific CD4+ T cells. They can recognize bacterial antigens and are often oligoclonal. The expanded clones may persist for several years [42, 136].

Approximately 5% of the CD3+ cells in all organized lymphoid organs and also in the skin- and gut-associated lymphoid tissues express TCRγδ molecules [66]. Nearly all γδT cells lack expression of CD4 and most lack CD8 or express it at relatively low levels. In contrast to the αβ T cells no common restriction element such as MHC is presently known for γδ T cells. They represent a small subpopulation of the CD3+ T cells in the human gut where they are randomly distributed within both the epithelium and the lamina propria [18]. TCRγδ T cells are preferentially located in the splenic sinusoids while TCRαβ-bearing lymphocytes mostly occupy the periarteriolar sheaths of penicillary arteries [50]. The preferential homing of γδ T cells to the epidermis seen in the mouse, is not present in man but there are differences in migration of γδ versus αβ T cells [18].

Delicate and highly redundant control mechanisms keep the immune system alert against foreign proteins, neo-antigens, and microorganisms. The immune response also has to be self-limited and under tight control to avoid self-destruction of the host. Failure to keep this delicate balance can lead to autoimmune disease or malignant lymphoproliferations. Upon antigenic stimulation, a polyclonal or oligoclonal lymphoproliferation is generated. Clonal populations can emerge if immune surveillance fails to control the lymphoproliferation. Clonality then suggests autonomous tumour cell growth; an important though not absolute diagnostic criterion for malignant lymphomas.

Southern blot analysis of T cell receptor gene rearrangements

Investigations of TCR rearrangements help to define clonality, lineage and the stage of differentiation of T lymphocytes. This approach is not only valuable for primary diagnosis. It can also successfully monitor disease spread, identify early relapse and recognize phenotypic changes of the tumour cells as part of their clonal evolution. Southern blot analysis has proven to be diagnostically important in situations where only small samples are available and immunohistochemical studies are difficult to interpret. Cytological specimens of lymphoproliferative disorders or unclassified large cell tumours and small biopsy specimens from gastrointestinal mucosa or skin are examples [64, 86, 133, 181].

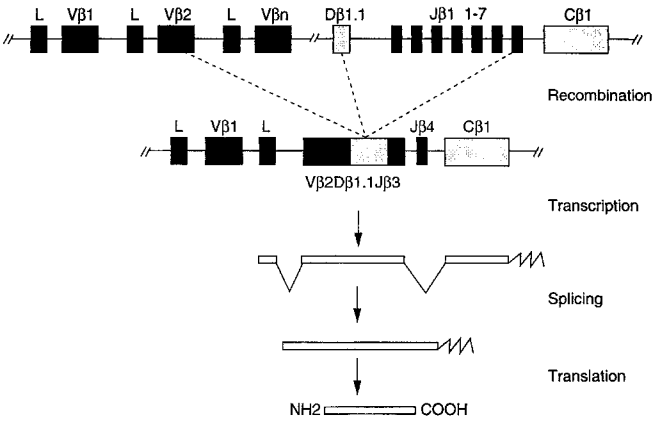

Fig. 3 Rearrangement of the TCR β chain genes. Exons encoding the different *V*, *D* and *J* regions are separated from each other and the *C* region gene by intron sequences. Leader sequences (*L*) are indicated 5′ of the *V* segments. Joining of individual *V*, *D*, and *J* sequences in a developmentally driven recombination process results in the loss of intervening chromosomal DNA. Restriction sites, normally present in germ line configuration, are removed and new sites introduced in the rearranged fragment. The newly assembled gene is transcribed to RNA and intron sequences are spliced out bringing the VDJ and C regions next to each other. This provides the template for translation into the receptor chain protein

Southern blotting is the most reliable technique to detect clonal rearrangements in T cell tumours. DNA extracted from cell suspension is digested with restriction enzymes which cut the double-stranded DNA molecules within specific recognition sequences throughout the genome. This invariably produces fragments or characteristic size in germ-line DNA, but rearrangement events – mostly accomplished by deletion processes – alter the distance between and presence of the original restriction sites (Fig. 3). Therefore, the germ-line fragment is replaced by a fragment of different size. Restriction fragments are size-separated by gel electrophoresis and then transferred to membranes. The membrane-bound DNA fragments are hybridized with radioactive-labelled TCR gene probes and autoradiography is done. Rearrangements in polyclonal lymphocyte populations generate many thousands of individually sized fragments differing from the germ-line configuration. This results in a vertical smudge in the autoradiogram upon DNA hybridization with labelled Ig or TCR gene probes. If more than 5% of a given cell population contain the same (clonal) rearrangement a distinct new band becomes detectable

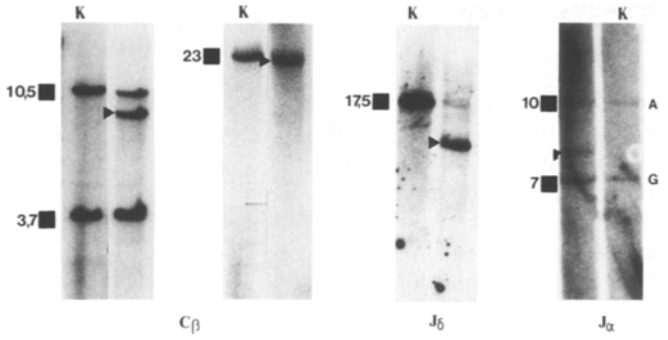

Fig. 4 Southern blot detection of TCR gene rearrangements in lymphoepithelioid (Lennert's) lymphoma. DNA, probed with a TCRβ constant region gene fragment (*Cβ*), was cut with *Eco*RI (left) or *Bam*HI (right). *Bam*HI-digested DNA was probed with a TCRδ joining region probe (*Jδ*) and *Xba*I-restricted DNA hybridized with two TCRα J-region probes (*A*, *G*). The size of the germline fragments of control DNA (*K*) is given in kilobasepairs, the new clonal bands in the lymphoma sample are marked by *arrowheads*

(Fig. 4). The sensitivity in leukaemia studies can even be lower than 5% if only few polyclonal T lymphocytes accompany the malignant clone and DNA degradation is kept to a minimum. DNA degradation is the limiting factor in the analysis of DNA extracted from paraffin-embedded tissue specimens.

TCR gene probes

Rearrangements of the TCRβ are normally examined with cDNA probes containing the Cβ1 or Cβ2 gene segment [155, 187]. They detect both constant region genes of the TCRβ chain. Usually DNA is examined after separate restriction with each of the three enzymes *Bam*HI, *Eco*RI, and *Hin*dIII, which results in fragments of specific size in germline DNA carrying the C region genes. Due to a partial resistance of one of the restriction sites, *Eco*RI digestion occasionally produces an additional 7.9 kb fragment that should not be mistaken as evidence for clonal rearrangement [56]. If a rearranged band is present it should be detectable in *Bam*HI as well as *Eco*RI or *Hin*DIII digested DNA samples. Probes directed against the Jβ2 gene cluster detect a rearrangement involving the Cβ2 gene in *Eco*RI-cut DNA [47].

Most investigations of the TCRγ chain are performed using a genomic Jγ gene probe that hybridizes to the first and the second Jγ cluster [105, 138]. In *Eco*RI digested DNA the Jγ2.3 gene sometimes resides on the same 1.8 kb fragment as the Jγ1.3 gene instead of the germline 3.3 kb fragment due to the presence of a polymorphic *Eco*RI restriction site [55]. The TCRγ gene differs from the β gene in its very limited number of rearranging Vγ genes. They give rise to only a few non-germline bands of distinct molecular weight in polyclonal T cells by DNA hybridization studies [171]. T cell clones with TCRγ rearrangement may be masked by contaminating polyclonal T cells. Alternatively, different intensities of non-germline bands produced by polyclonal T cells may produce one predominant rearranged band that can be mistaken as evidence for a clonal lymphocyte population. For these reasons it is most helpful to evaluate TCRγ gene rearrangements together with the TCRβ gene configuration. The Cγ2-region probe detects all rearrangements involving both Jγ-gene clusters. It hybridizes to a 20 kb-long *Bam*HI fragment containing the Jγ1 and a 12.5 kb fragment containing the Jγ2 gene segments [138, 189].

TCRδ probes come from genomic DNA sequences containing Jδ1 or Jδ2 regions, or Cδ sequences [8, 163]. They detect all the rearrangements in this gene locus when used with *Bam*HI-restricted DNA. However, in many mature T cell neoplasms this gene locus is deleted on one or both alleles which results in a diminished intensity or absence of the germline band. This decrease in hybridization intensity is hidden, however, if the clonal T cell population is small and many polyclonal T cells, B lymphocytes and non-lymphoid cells are present in the sample.

Cα gene probes are of limited use for the evaluation of the TCRα chain gene recombinations. The Jα regions are spread over a distance of about 85 kb [62]. This area can be covered with genomic DNA clones obtained from chromosomal walking [8, 28, 81]. They are useful probes for DNA cut with several different restriction enzymes. An *Eco*RI-RFLP for a Jα-probe mapped approximately 55 kb 5′ of the Cα gene has been described. It results in either a 7 kb (80% of cases) or a 14 kb germline *Eco*RI fragment [109]. In one study, 61 lymphoma samples were investigated for the TCRα chain gene configuration [59]. Eight different genomic J region probes were used [28]. An additional 16 kb non-germline band occurred in 15% of non-T cell lymphomas as well as inflammatory lymph node lesions when the JαD probe was used on *Eco*RI-digested DNA. This may represent an RFLP or a partially resistant restriction site.

Southern blot analysis of TCR genes has helped to understand intestinal large cell anaplastic lymphomas (LCAL), lymphoepitheloid (Lennert's) lymphomas (Fig. 4) and angioimmunoblastic lymphadenopathy-like (T/AILD) T cell lymphomas concerning their lineage and clonality [61, 80, 110, 131]. The combination of molecular genetic data, immunohistochemical findings and cytomorphology helped to recognize these disorders as specific lymphoma subtypes. Immune receptor gene analyses are of limited value in the differential diagnosis of Hodgkin's disease (HD) versus non-Hodgkin's lymphoma in morphologically equivocal cases. Immunogenotyping sometimes may not help in differential diagnosis between peripheral T cell lymphoma with atypical large T blasts and HD (usually of mixed-cellularity subtype or unclassified): TCRβ rearrangements have been observed in HD as well [60, 75, 144]. The separation of HD from non-Hodgkin's lymphoma thus remains impossible at times.

The TCRβ genes have been studied most extensively and rearrangement analyses with such probes have been

shown to provide reliable results. In several large series encompassing more than 250 B cell and 80 T cell lymphomas [31, 35, 74, 180], all cases of B cell lymphoma had clonal IgH rearrangements and TCRβ recombinations occurred in 94% of the T cell neoplasms. In 4% of the clonal B cell disorders an additional TCRβ rearrangement was found, likely representing a cross-lineage rearrangement. As part of another large retrospective study comparing lymphoma genotype and phenotype, 97 T cell lymphomas were studied for rearrangements of all four TCR chains [59]. Clonality was confirmed in nearly all T cell lymphomas except 3 of 48 T/AILD lymphomas. Clinically these 3 lymphomas behaved as malignant as those with detectable rearrangement [154]. Probes against the TCRβ constant region gene detected clonality in 97% of T lymphomas. TCRγ gene probes showed rearrangements in 92% of the samples. The use of these probes should be combined with the investigation of the TCRβ gene structure to avoid false-positive results due to pseudoclonal TCRγ rearrangement pattern in poly- or oligoclonal T cell populations [171]. TCRα rearrangements were detectable in only 82% of the T cell lymphomas investigated with eight Jα region gene probes and after DNA digestion with any of four different restriction enzymes [59]. TCRα gene rearrangement studies thus are not for routine purposes. The presence of a clonal TCRα and β rearrangement in two LCALs without lineage-restricted surface marker expression suggested that both were mature T cell neoplasms. Only 65% of peripheral T cell lymphomas have detectable TCRδ rearrangements or TCRδ deletions [90, 96, 163]. This is mainly because of the TCRδ locus deletions during the developmentally regulated TCRα rearrangements. The deletions are only detectable if the tumour sample consists mainly of clonal T cells. In acute lymphoblastic leukaemias of T-type (T-ALL) or T-lymphoblastic lymphomas TCRδ rearrangements occur in more than 60% of the cases [57, 71].

Aberrant Ig gene rearrangements in T cell lymphoma

The frequency of illegitimate Ig gene rearrangements in mature T cell neoplasms of the lymph node is around 10% for most morphological subtypes. In a large series of T cell lymphomas, however, 12 of 46 T/AILD lymphomas had illegitimate IgH gene rearrangements [59]. Rearrangement analysis of additional T and B cell receptor genes helped to identify these recombinations as aberrant. Rearrangement of the IgH and the Igκ genes was exclusively observed in clonal B but not in clonal T cell proliferations. Exceptions from this rule are very rare and mainly restricted to T-ALL [68]. IgH cross-lineage rearrangements are mostly monoallelic and the rearranged bands often have a molecular weight that is lower than in B cell lymphomas [59]. Cross-lineage rearrangements probably arise in transformed lymphoid cells unrelated to the ontogenetic program of lymphocyte development either by aberrant activation of the recombinase machinery or as part of chromosomal abnormalities involving immune receptor gene loci. Additional analyses of TCRγ, δ, and α as well as Ig light chain genes may identify ontogenetically programmed rearrangements of clonal T and B cell populations coexisting in the same tumour tissue. Such a bi-lineage lymphoma involvement occasionally is observed in T/AILD lymphomas [58, 92, 110]. Aberrant rearrangements of several TCR genes are rather frequent only in pre-B cell neoplasms [57, 161]. Proper lineage assignment absolutely requires immunophenotyping in these cases.

T/AILD lymphomas

T/AILD lymphomas may not only present with aberrant IgH rearrangements but sometimes also contain more than two rearranged fragments of a TCR gene. This suggests the presence of oligoclonal lymphocyte populations [59, 110]. A clonal lymphocyte proliferation may not originate from a single transformed T cell in certain lymphoma subtypes. It is possible that several clones emerge from an activated polyclonal lymphocyte pool forming a predominant oligoclonal population. Eventually monoclonal disease can develop through a selection advantage for one of these clones.

Cutaneous T cell lymphoma

The variable histological picture of cutaneous T cell disorders is reflected by the genotypic heterogeneity detected in DNA samples from such lesions. T cell clonality does not necessarily suggest a malignant course of disease. Several groups reported clonal and sometimes oligoclonal TCR gene rearrangements in lesions of lymphomatoid papulosis (LYP), pityriasis lichenoides et varioliformis acuta, pagetoid reticulosis and granulomatous slack skin disease [82, 103, 176, 177, 182]. There is little doubt, however, that these T cell proliferations are abnormal and that some predispose to the development of clinically malignant lymphoma. The detection of clonality may indicate a prelymphomatous stage of disease which is still partially controlled by the immune system. Rearrangement studies of cutaneous T-cell rich lymphoproliferative disorders of the skin thus are diagnostically helpful in discriminating between a merely inflammatory lesion and abnormal lymphoproliferation [64]. Heterogeneity of gene rearrangement patterns is seen in established malignant cutaneous T cell lymphomas such as mycosis fungoides (MF) or Sézary syndrome. The oligoclonality and the cross-lineage IgH rearrangements sometimes found in these skin lymphomas is reminiscent of the situation in T/AILD lymphomas [11, 173, 175]. MF stage I or II disease frequently lacks clonal TCR rearrangements by Southern blot or PCR [44, 139, 156]. In early MF lesions the malignant clone may be very small and form less than 1–5% of the infiltrating T cells. It is also possible that MF starts with an expansion of many

small T cell clones. One of these eventually gains a selection advantage over the other clones during tumour progression. MF involvement of enlarged lymph nodes can be detected by clonal TCR rearrangements. Its presence is associated with an inferior survival [115].

Malignant lymphomas of γδ T cells

Expression of the γδ TCR is not infrequent among T-ALLs [43] but exceedingly rare in post-thymic T cell lymphomas. These can present as hepatosplenic T cell lymphomas, as large granular lymphocyte (LGL) leukaemia, and rarely as cutaneous lymphoma [23, 51, 54]. Some of these lymphomas have their TCRβ genes rearranged besides the δ and γ genes. Clonal TCRδ gene rearrangements without detectable TCRβ gene recombination also occur in lymphoma samples which lack γδ surface expression]96]. This could partly be explained by chromosomal translocations involving the TCRαδ gene locus in transformed T cells that may have matured extrathymically.

LGL leukaemia

Combined immunophenotypic and genotypic investigations of LGL leukaemia have established their origin from two separate lineages [113]. LGL leukaemia originating from CD3+ activated cytotoxic T cells have clonally rearranged their TCRβ genes and express the αβ TCR or more rarely express the γδ TCR. In the latter case TCRβ genes may have a germline configuration but TCRγ genes are clonally rearranged [54, 158]. Leukaemias derived from natural killer cells do not express CD3 or TCR, and lack TCR rearrangements. The course of disease is progressive in CD3–LGL leukaemia subtypes but usually chronic in CD3+ variants. CD3–LGL leukaemias occasionally have detectable clonal TCR rearrangements suggesting that they are activated mature T cell leukaemias with CD3 antigen loss [142].

PCR analysis of T cell receptor gene rearrangements

PCR-based rearrangement detection initially focussed on the detection of minimal residual disease (MRD) in ALL patients [37, 69, 116]. Particularly the TCRδ locus with its limited numbers of V and J region genes was used to design V and J region specific primers. The length of the amplification products is variable among individual rearranged fragments because of the extensive junctional diversity. TCRδ gene sequences are not routinely amplified in peripheral T cell lymphomas because of the frequent TCRδ gene deletions in mature T cells. PCR detection of clonal rearrangements in T cell lymphomas mainly has been done with TCRγ-specific oligonucleotides. The limited repertoire of these genes allows the use of primer mixes in a so-called multiplex

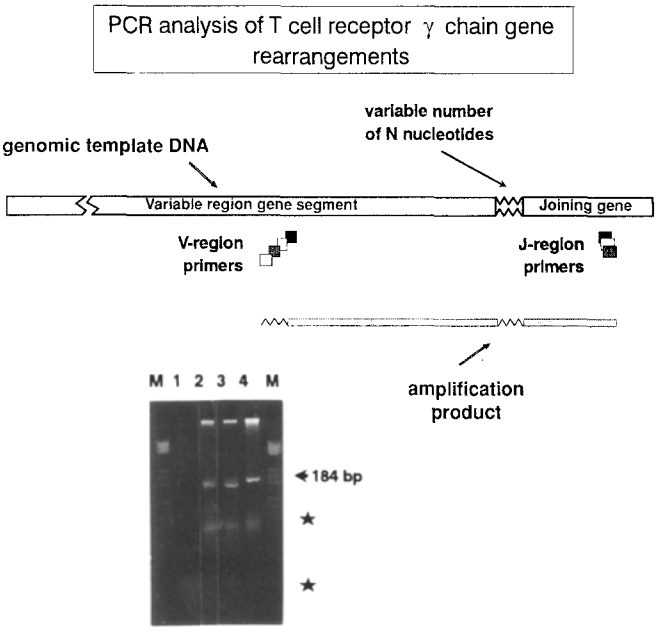

Fig. 5 Polymerase chain reaction (PCR) detection of TCRγ gene rearrangements. Several primers for *V* and *J* regions are used in a multiplex PCR. The amplification products from individual rearrangements differ in size because of the variable length of the *N* segment and of the different amplified *V* region gene sequences. The TCRγ PCR result is shown. Amplification products are separated on an ethidium bromide-stained 3% agarose gel. Lane 1 contains no template DNA and lane 2 shows a polyclonal smear amplified from a sample of nonspecific lymphadenitis. Clonal amplification products are shown in lane 3 (from an angioimmunoblastic lymphoadenopathy-like T cell lymphoma) and lane 4 (positive control DNA from a T-lymphoblastic lymphoma). Lane *M* contains the molecular weight marker and primers and primer-dimer artifacts are indicated by *stars*

PCR which identifies most rearrangements of the γ gene [168]. Visualization of the amplification products on a polyacrylamide gel is normally sufficient to identify uniformly sized clonal amplification products. Rarely is Southern blot analysis or sequencing of the gene products necessary. Sophisticated separation techniques such as denaturing gradient or temperature gradient gel electrophoresis (DGGE or TGGE) are invaluable in cases where minor clonal bands have to be detected among polyclonal amplification products [19, 65]. Like Southern blot analyses, polyclonal T cells generate a background smear since the length of the amplified fragment varies between individual T cells due to the different V gene usage and diversity of the flanking N regions (Fig. 5). The sensitivity for clonality detection does not, therefore, differ between Southern blot and PCR if the amplification products are separated by non-DGGE. The PCR template DNA, however, does not need to be present in large quantities (less than 1 μg usually is sufficient). Degradation of the DNA from formalin-fixed, paraffin-embedded material, archival air-dried blood or bone marrow smears does not compromise the reaction. Prolonged formalin fixation over a week results in less reliable amplification when compared to shorter fixation times [143]. Certain fixatives, like Bouin's solution or

B5 [123], or decalcification of tissue with ethylenediamine tetraacetic acid causes such extensive DNA degradation that at best only very short sequences can be amplified. Another advantage of the PCR approach is the possibility to target certain small tumour-infiltrated areas on stained sections. These areas can be scraped off the slide and used as a source for template DNA [179]. Alternatively, this region can be shielded against ultraviolet radiation which denatures the DNA in the surrounding tissue [151]. Only the DNA from the protected areas of the slide remains amplifiable.

PCR primers

A multiplex PCR for TCRγ rearrangements can be performed using a mixture of different V and J region-specific primers [168]. The use of consensus primers [119] requires differential cutting of the amplification products with restriction enzymes [162] or separation by DGGE [19].

Several primers have been developed for the TCRβ PCR which recognize a set of different V, D and J region genes [118]. Even though clonal TCRβ gene rearrangements are missed in many cases this method appears a useful supplementary test for the assessment of clonally expanded T cells expressing the αβ heterodimer.

TCRδ primers have widely been used for the detection of clone-specific rearrangements in ALL [69, 116]. Primers established from the unique sequences of the VDJ or VDDJ junctions are clone-specific and their use considerably enhances the sensitivity of MRD detection. With such a clone-specific PCR the usual sensitivity level of 1 in 10^6cells can be reached [147].

Consensus Vα and Jα region primers for the amplification of TCRα gene recombinations are not available. A so-called reverse transcription (RT)-PCR can be done with reverse transcribed cellular RNA using C region oligonucleotides and degenerate 5′ end primers for the cDNAs [21]. A predominant, clonal rearrangement is detected by cloning and sequencing of the amplification products or by separation of the fragments according to their conformation usually on a gradient gel or by single-strand conformation polymorphism analysis. The PCR procedures for TCRα gene rearrangement detection are more sophisticated than TCRγ/δ PCRs and may not be widely applicable for routine diagnostic purposes.

In the past 5 years, considerable experience with PCR detection of T cell clonality has accumulated. Investigations started with the search for MRD in ALL samples [37, 69, 185]. A positive result in bone marrow-derived DNA was found to have clinical relevance in predicting relapse [127]. Later, TCRγ PCR studies of nearly 70 T cell lymphoma cases have been reported. They found clonality in 87% of the lymphoma samples with no false-positive results [58, 119, 168]. Cross-lineage rearrangements may be detectable by PCR in cases of lymphoblastic lymphoma with a pre-B phenotype [167]. A recent study on gastrointestinal LCAL has shown that TCRγ-PCR helps to identify T lineage of tumour cells lacking surface expression of T cell markers in routinely-fixed samples [65]. Clonal TCRβ gene recombinations have been detected in 13 of 16 T cell lymphoma specimens by PCR [118]. Combined investigation of T cell neoplasms with amplimers for both, TCRγ and TCRβ, may lead to superior results than the use of either of the primer sets alone. The fast and simple PCR analysis of DNA samples has priority over Southern blot procedures in diagnostically difficult cases. Because of the higher rate of false-negative results with PCR, Southern blot studies are not obsolete and are required when PCR results are unsatisfactory.

Limitations of rearrangement studies for clonality

Molecular genetical studies require a dedicated laboratory with sufficient volume of samples and significant test experience. Failure to detect clonality, or the finding of unexpected or illegitimate TCR rearrangements is rare under these circumstances. Diagnostic problems arise particularly when molecular results are not correlated with histological and immunophenotypic findings. It also helps to limit molecular studies to diagnostically challenging cases or the search for minimal disease. PCR techniques are very suitable for MRD evaluation and likely of prognostic value in ALL patients [127]. The detection of circulating tumour cells, however, appears clinically insignificant for other lymphoma patients in complete remission [77].

Absence of expected T cell receptor rearrangements

Failure to detect clonal rearrangements can have technical reasons. A clonal cell population may not be well represented in the specimen used for DNA extraction; the DNA may be degraded and not suitable for the detection of high-molecular weight fragments in Southern blot analysis; DNA preparations from routinely processed tissue may contain PCR inhibitors. Some T/AILD lymphomas and stage I and II MF may not contain molecularly detectable T cell clones as discussed earlier. The absence of a clonal gene rearrangement in the presence of a histological picture typical for malignant lymphoma is diagnostically irrelevant. Examinations can be repeated in subsequent biopsies or specimens taken from a different site for molecular genetic support of the diagnosis. In some lymphoma subclasses TCR gene rearrangement studies may fail to provide evidence for clonality. Cases of immature T cell neoplasms like some T lymphoblastic lymphomas or T-ALL [38, 61, 130] and a subclass of CD3-nodal and extranodal nasopharyngeal T cell lymphomas lack TCRγ and β rearrangements [96, 178]. It is still under investigation whether some of these neoplasms belong to the natural killer cell lineage.

Unexpected T cell receptor rearrangements

Clonal TCR rearrangements are occasionally found in non-T cell neoplasms. This is rarely explained by chromosomal translocations involving a TCR gene locus. More frequently it may originate from an error in the recombinase system acting on germline TCR genes in malignant myeloid cells or normal activated or transformed B lymphocytes. Aberrant, non-functional TCR transcripts have been found in mature tonsillar T cells and may be initiated in activated B cells during the Ig class-switch [26, 146]. Aberrant TCR rearrangements, mainly of the β chain genes, occur in up to 10% of mature B cell neoplasms. These B cell lymphomas nearly always have their TCRγ genes in germ line configuration in contrast to the lineage-specific TCRγ and β rearrangement status in T cell neoplasms [1, 32]. In B-LCAL and centrocytic lymphomas aberrant TCR gene rearrangements have been detected more frequently than in other B cell lymphomas [52, 63, 75]. In common ALLs and pre-B lymphoblastic lymphomas several TCR loci frequently undergo rearrangement besides the Ig genes. This renders genotypic lineage determination by Southern blot procedures of PCR unreliable [70, 71, 72, 161]. Cross-lineage TCR rearrangements are also detected in acute leukaemias, especially those with TdT expression. The TCRβ genes are most often affected and simultaneous aberrant recombinations of several TCR gene loci in the myeloid cell clone are exceptional [5, 34, 104, 150]. The occurrence of clonal TCR rearrangements is diagnostically challenging in lymphoproliferative T cell disorders which are clinically considered non-malignant. Activated T cell clones especially in lymphoproliferations of the skin may expand to a point where they become detectable by rearrangement studies but remain localized and controlled by the immune system. Clonal cutaneous lymphoproliferations such as LYP lesions, however, may coincide with or transform into a malignant T cell lymphoma [82, 176]. A clonal TCR rearrangement thus may not help in the differential diagnosis between LYP and a cutaneous LCAL. T lymphocyte clones may also expand due to persistent antigen exposure [42, 88, 111]. These lymphoproliferations are mainly oligoclonal but it was recently shown that single reactive cytotoxic T cell clones are detectable in healthy, elderly individuals [137]. A similar clonal selection mechanism could have caused the abnormal finding of a clonal TCRβ gene rearrangement in a sample of hyperplastic lymphadenopathy associated with lung cancer [29].

Translocations involving T cell receptor gene loci

The analysis of recurrent chromosomal abnormalities is another approach to the detection of clonality in lymphoproliferations and provides unique markers for the clonal population. Moreover, cloning and molecular analyses of the breakpoints provide important biological information about lymphomagenesis and may help to delineate T cell lymphoma subtypes. This approach has been successful in B cell lymphoma, where the presence of *bcl*-1 or *bcl*-2 translocations are characteristic for centrocytic or centroblastic/centrocytic lymphomas, respectively. It also supports the previous cytomorphological characterization as separate entities [108].

Chromosomal aberrations have been characterized predominantly for T cell leukaemias. The most frequently affected chromosomal site in these neoplasms is the TCR α/δ gene locus on chromosome 14q11. Involvement of the TCR β chain genes on chromosome 7q35 occurs more rarely and the TCR γ genes on chromosome 7 mainly participate in chromosomal rearrangements of T cells from ataxia telangiectasia patients [6]. The foreign genetic material, which joins the TCR gene loci, comes from a variety of other chromosomes. Frequently sequences coding for various transcription factors (TFs) are affected. As discussed later, many of these TFs are physiologically expressed in non-lymphoid cells [128]. Abnormally expressed in thymocytes and T cells they may act totally independent of regulatory pathways and contribute to malignant transformation.

Involvement of either δ or α TCR genes in chromosomal translocations is possibly the consequence of abnormal rearrangement processes at an early or late thymocyte stage, respectively. Participation of the VDJ recombinase system is likely since the breaks often occur at heptamer recognition sequences of TCR D or J segments. This is obvious in the translocation t(14;14) (q11;q32) of the T-ALL cell line SUP-T1 where two rearranging genes (TCR α and IgH) from opposite chromosomal ends of the long arm are fused [7]. The translocation t(14;14) in cases of T-prolymphocytic leukaemia or chronic lymphocytic leukaemia of T-type, however, has been described to involve a chromosomal region distant from the IgH gene locus at 14q32.1 rather than 14q32.3 [39, 122]. Heptamer-like recognition sequences are sometimes also found around the TF gene sequences involved in the translocation. This suggests that an erroneous recombinase activation may have contributed to chromosomal breakage outside the immune receptor gene loci [14].

Most TFs known to play a role in chromosomal translocations involving TCR gene loci can be grouped according to their binding motifs. Genes for LYL-1 (chromosome 19p13) [121] and TAL-1 (1p32) [30] encode early haematopoietic TFs. Both contain a helix-loop-helix (HLH) motif that mediates protein dimerization and interacts with target DNA sequences [172]. The TAL-1 gene translocation t(1;14) (q32;q11) is present in about 3% of T-ALLs but heptamer-mediated deletional rearrangements in its 5′ flanking region occur in up to 25% of T-ALLs [4, 22]. Another gene encoding an HLH motif, the c-*myc* gene, takes part in chromosomal translocations of T-ALLs [10, 152]. In the t(8;14) (q24;q11) abnormality either the JαCα or the Jδ-Cδ-Jα-Cα region is moved to a region 3′ to the c-*myc* gene on the 8q+

chromosome. The closely sequence-related genes for TTG-1 and TTG-2 on chromosome 11p15 and 11p13, respectively, contain a zinc finger-like protein dimerization motif called LIM domain [15]. Both factors are physiologically expressed predominantly in neural and other non-T lymphoid tissues [120, 145]. The third kind of TF is the homeobox gene HOX 11 on chromosome 10 which participates in the translocation t(10;14)(q24;q11) [114]. This abnormality occurs in approximately 7% of T-ALLs [45, 46]. HOX 11 is normally transcribed in liver tissue but not in haematopoietic cells. A variant translocation t(7;10) (q35;q24) involving the TCR β gene locus probably results from aberrant recombination processes [87].

The non-receptor tyrosine kinase $p56^{LCK}$ is important for the signal transduction in T cells. The LCK gene participates in the translocation t(1;7) (p34;q35) of the T-ALL derived cell lines HSB-2 and SUP-T12 [169]. Analysis of the breakpoint site suggested that the break occurred during a failed attempt of Vβ to DJβ joining. No ectopic heptamer-like sequences were found close to the other side of the breakpoint.

Translocations in T cell leukaemias involving TCR and TF genes may result from accidental recombinase activities. They largely occur in thymic development of T cells and may provide leukaemogenic potential. This could explain why T cell leukaemias are mostly involved by these chromosomal abnormalities rather than peripheral T cell lymphomas where TCR genes remain mostly unaffected [79, 101, 125]. In the latter instance, mechanisms other than TF translocations may initiate disease and the chromosomal changes could arise later in disease progression, independent of the initial transformation event.

An example for a non-random translocation in post-thymic T cell neoplasms is the t(2;5) (p23q35) abnormality which is characteristic of T-LCAL [12]. The sequences coding for the intracellular domains of an insulin receptor-like kinase gene on chromosome 2, termed ALK (for anaplastic lymphoma kinase), is fused with the nucleophosmin (NPM) gene on chromosome 5 [126]. Regulatory sequences of the NPM gene, which is transcriptionally most active before the cell entry into the S phase, may activate the ALK gene. This kinase gene is physiologically not transcribed in lymphocytes. The fusion protein of the two genes could potentially phosphorylate intracellular substrates which are normally under control of lymphoid lineage-specific kinases only.

The t(4;16) translocation was cloned from the tumour cell clone of an enteropathy-associated intestinal T cell lymphoma. In this abnormality the interleukin-2 gene on chromosome 4q26 had recombined with a gene on chromosome 16p13 termed B-cell maturation (BCM) gene [98]. A fusion transcript was not detected, however, and the importance of this translocation for lymphogenesis remains to be shown.

Molecular detection methods for chromosomal translocations

Molecular probes are generated through isolation and sequence analysis of regions flanking the chromosomal breakpoints. These probes are useful for the detection of chromosomal translocations in Southern blot analyses if the breakpoints are clustered and not spread out over a large chromosomal region. After identification and cloning of specific chromosomal breaks their sequences can be analysed and tumour-specific PCR primers flanking the breakpoint be designed. This PCR approach is highly suitable for molecular follow-up studies in a particular patient since amplification products are only generated from cellular DNA carrying this translocation [83]. Fusion gene transcripts from reciprocal chromosomal translocations are detectable by RT-PCR.

This technique has been applied to search for the translocation t(2;5) in T-LCAL [126]. Another fast diagnostic approach is the detection of translocations by non-radioactive, usually fluorescence-based, in situ hybridization of chromosomes (FISH) [135]. The probes detect chromosomal DNA either from metaphase spreads or non-mitotic cells [36]. The latter so-called interphase cytogenetic analysis can be done even on previously stained cells [3]. Simultaneous immunophenotyping of the cells helps to focus the interpretation of the results on the tumour cell population [174]. Genomic DNA probes flanking the breakpoint on both sides and derived from different chromosomes are labelled with two different fluorochromes. The two signals would be located on different chromosomes in a metaphase spread or found far apart in interphase cells if the translocation is absent. Joining of genetic material from the two chromosomal localizations, in contrast, leads to a doublet fluorescence signal after hybridization [164].

Results from FISH analysis are usually available within 2 days, which compares favourably with the Southern blot procedures. With more translocation-specific probes available, molecular cytogenetics promises to be a fast and simple technique for the detection of non-random chromosomal abnormalities. Though less sensitive than PCR detection, FISH is potentially of higher specificity in tracing single tumour cells carrying a specific anomaly. The hybridization efficiency of FISH probes in sections from archival material depends on the fixative and the age of the paraffin blocks. A negative result in these instances is diagnostically not helpful. RT-PCR analysis for the identification of chromosomal translocations generally requires fresh-frozen tissue or cell suspensions. RNA extracted from routinely-processed specimens is strongly degraded and the reverse transcribed cDNA fragments are of insufficient length for efficient amplification of more than 200 bp products.

Conclusion

The discovery of immune receptor gene rearrangements and their developmental regulation has provided a new

perspective for clinical immunology and haematology. Questions arising from cytomorphological observations and immunocytochemical studies regarding clonality, cellular lineage and abnormal activation of T cells can now be addressed at a molecular genetical level. TCR gene rearrangement studies have proven to be reliable for the clonality assessment of T cell proliferations. Molecular genetic analyses also help in lineage determination and identification of the lymphoid cell differentiation stage. It now has become routine for laboratories committed to the diagnosis of lymphoproliferative disorders. The simple and timesaving PCR approach has been added to the molecular genetical repertoire. This technique requires only small amounts of DNA for determination of clonality and lymphocyte lineage and is applicable to DNA extracted from paraffin-embedded tissues. PCR for MRD detection in ALLs and for clonality studies in T cell lymphomas are very effective. Limitations of the molecular techniques are well recognized and can be handled in an experienced laboratory. If studied in the context of morphology and immunohistochemistry, molecular genetic data are of great diagnostic and sometimes prognostic value.

Although Southern blot analyses helped to identify certain T cell proliferations as malignant lymphomas, this technique has not provided new criteria for a biological subclassification of T cell lymphomas. T/AILD lymphomas have characteristic rearrangement profiles, but they are not unique to this malignant T cell disorder. Molecular cytogenetics are about to become another powerful diagnostic tool. They provide not only sensitive and highly specific clonal markers but may also help to find the genetic basis for differences among T cell lymphomas reflecting their morphological and clinical characteristics. Certain non-random chromosomal abnormalities in T cell neoplasia seem to arise because of an aberrant recombinase activity in developing T cells. Investigations of these translocations have identified many new growth regulatory genes. Sets of molecular probes for the analysis of a variety of chromosomal breakpoints will soon be available. They can be used in FISH for rapid detection of abnormalities. TF genes often reside near the breakpoint region and are sometimes abnormally transcribed. Aberrant activation of nuclear TFs and protein tyrosine kinases during thymic ontogeny or antigenic T cell stimulation may lead to an altered intracellular signalling pathway and interfere with ordered T cell development, differentiation, and proliferation. This may either initiate malignant transformation or provide an activated T cell subpopulation with a selection advantage. Once the role of the T-cell lymphoma- and leukaemia-specific structural chromosomal abnormalities for malignant transformation is established, they are likely to become targets for specific therapeutic interventions aimed directly at the tumour cells.

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