HUMAN T CELL DIFFERENTIATION: BASIC ASPECTS AND THEIR CLINICAL APPLICATIONS

CIP-GEGEVENS KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Dongen, Jacobus Johannes Maria van

Human T cell differentiation: basic aspects and their clinical applications / Jacobus Johannes Maria van Dongen. - Rotterdam : Erasmus Universiteit, Afdeling Immunologie. - ill.
Proefschrift Rotterdam. - Met lit. opg.
ISBN 90-73436-02-8
SISO 608.3 UDC 612.017(043.3) NUGI 743
Trefw.: T-lymfocyten.

HUMAN T CELL DIFFERENTIATION

Basic aspects and their clinical applications

T-CEL DIFFERENTIATIE BIJ DE MENS

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof. Dr. C.J. Rijnvos
en volgens besluit van het College van Dekanen.
De openbare verdediging zal plaatsvinden op
woensdag 20 juni 1990 om 13.45 uur

door

Jacobus Johannes Maria van Dongen

geboren te Bergen op Zoom.

PROMOTIECOMMISSIE:

Promotoren:	Prof. Dr. R. Benner Prof. Dr. A. Hagemeijer
Overige leden:	Prof. Dr. B. Löwenberg Prof. Dr. J.J. van Rood

Dit proefschrift werd bewerkt binnen de vakgroep Immunologie en Immunohistologie van de Faculteit der Geneeskunde, Erasmus Universiteit, Rotterdam.

Het onderzoek werd mede mogelijk gemaakt door de Nederlandse Kankerbestrijding (Koningin Wilhelmina Fonds) en de actie "Geven voor Leven".

In de drukkosten van dit proefschrift werd bijgedragen door Becton Dickinson, De Beer Medicals B.V., Nordic Immunological Laboratories en Zeiss Nederland B.V.

Het proefschrift werd gedrukt door Haveka B.V. te Alblasserdam.

Aan Jeanette
Aan mijn ouders

HUMAN T CELL DIFFERENTIATION

Basic aspects and their clinical applications

CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

GENERAL INTRODUCTION

Immune recognition plays a central role in our understanding of the function of the immune system. The ability to specifically recognize foreign antigens allows selective but efficient actions of the immune system against all kinds of pathogens. This is mediated by antigen-specific receptors on B and T lymphocytes. Immunoglobulin (Ig) molecules represent the antigen-specific receptors of B lymphocytes, while the T cell receptor (TcR) has this function in T lymphocytes (1). Although these two types of antigen receptors have remarkable similarities in protein structure and their encoding genes, they differ significantly in their ability to interact with antigens (1). Via their surface membrane Ig (SmIg) molecules, B lymphocytes are able to recognize antigens in their native configuration either free in solution, on surfaces or on cell membranes (1). TcR molecules of T lymphocytes can only recognize processed or degraded antigens which are physically associated with major histocompatibility complex (MHC) molecules (2,3). This TcR-mediated recognition is therefore called MHC-restricted antigen recognition (2,3).

Expression of SmIg or TcR molecules by lymphocytes is acquired during lymphoid differentiation via several rearrangement processes in the Ig or TcR genes (3-7). B lymphopoiesis mainly occurs in the bone marrow (8), while the thymus is thought to represent the main tissue compartment for T lymphopoiesis (9-11). During T cell differentiation in the thymus the T lymphocytes are "educated" for their future functions, i.e. T cells which recognize self antigens are eliminated (negative selection), while positive selection occurs for T cells which recognize foreign (non-self) antigens in association with self-MHC molecules (12-14). Upon recognition of a TcR-compatible antigen, T lymphocytes are activated, start to proliferate and exhibit their regulatory or cytotoxic functions (2). These T cell functions play a central role in the regulation of the immune system. The T lymphocytes probably coordinate immune processes via cellular interactions and lymphokines and in this way adjust and harmonize the actions of the immune system.

The TcR consists of two chains, which are closely associated with the CD3 protein complex (TcR-CD3). The CD3 chains probably play an important role in signal transduction from the TcR to the cytoplasm (15). Two main types of TcR have been identified: the classical TcR-$\alpha\beta$ and the alternative TcR-$\gamma\delta$ (2,3,7). Data about the structure and rearrangement of the four TcR genes have become available during the last six years. The majority of these data are derived from *in vivo* and *in vitro* studies in mice (7,9). Experimental data on human T cell differentiation and human T cell function are mainly restricted to studies on thymocytes and T cell malignancies (10,11,16).

We have used freshly obtained cell samples from healthy individuals as well as cell samples

from leukemia patients and immunodeficiency patients to study human T cells and human T cell differentiation. Although most of these studies have a descriptive and inventory character, they reveal important information on the differentiation of T cells including the rearrangement and expression of TcR genes during their differentiation. Our studies also demonstrate that such basic information can be used for the development of new diagnostic tools. Optimal results in such studies can only be obtained, if a close collaboration is established between the clinical scientists and the laboratory scientists. Such a collaboration enables integration of the obtained results and evaluation of the sensitivity and specificity of newly developed diagnostic assays.

The following Chapters of this thesis describe the applied techniques (Chapters 2 and 3), the experimental work on human T cells and T cell differentiation (Chapter 4) and the application of the obtained information and techniques for diagnostic purposes (Chapters 5 and 6).

Chapter 2 summarizes the immunologic markers, which are generally used for immunophenotyping of leukocytes. The microscopic techniques to detect the expression of these markers are discussed. Especially the application of immunofluorescence labeling techniques are described, because they can easily be used for double or even triple labelings, which allow detailed analysis of specific cell populations as well as the detection of small cell populations with a specific immunophenotype.

Chapter 3 describes the genetic basis of the diversity of the antigen-specific receptors of lymphocytes, the Ig and TcR molecules. Also the differences between Ig and TcR molecules are discussed. Subsequently the Southern blot techniques for analysis of Ig and TcR genes are described. The pitfalls and limitations of these Southern blot analyses are indicated. The various aspects of these analyses are illustrated with a series of figures and special attention is paid to the germline configuration of the Ig and TcR genes.

Chapter 4 is a compilation of a series of studies concerning the heterogeneity of human T cells and human T cell differentiation. These studies concern the immunophenotype of T cells during T cell development and the hierarchic order of TcR gene rearrangements during T cell differentiation as studied in T cell leukemias. Also the expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ molecules in healthy individuals and children with hypoplasia or aplasia of the thymus is described. Based on these data the various processes during T cell differentiation and their thymus dependency are discussed.

Chapter 5 summarizes the application of immunologic marker analysis of leukocytes for diagnostic purposes. This Chapter focusses on applications for diagnosing leukemias and malignant lymphomas. The major achievements are the detailed and reproducible immunologic classification of the hematopoietic malignancies, the development of new immunologic markers such as the cytoplasmic expression of CD3 molecules (CyCD3) as marker for immature T cell leukemias and, most importantly, the detection of minimal residual disease. Extremely low detection limits for identification of malignant cells could be achieved in patients with a terminal deoxynucleotidyl transferase positive (TdT^+) T cell malignancy. Furthermore, early detection

of central nervous system leukemia has proven to be possible in patients with a TdT^+ malignancy.

Chapter 6 describes the occurrence of Ig and TcR gene rearrangements in lymphoid leukemias and malignant lymphomas. Analysis of Ig and TcR genes can be used for diagnostic purposes in patients with lymphoproliferative diseases. This is based on the fact that the far majority of malignant lymphoproliferations have clonally rearranged Ig and/or TcR genes, which can be detected by Southern blot analysis. It is emphasized that clonal Ig and TcR gene rearrangements may also occur in clinically benign lymphoproliferations, which implies that clonality per se is not equivalent to malignancy. A series of clinical cases is presented to illustrate diagnostic applications of Ig and TcR gene analysis.

Chapter 7 discusses the significance of the presented experimental data and their clinical application. In addition, some new developments are discussed, such as the application of the polymerase chain reaction (PCR) analysis of TcR-γ and TcR-δ genes for the detection of minimal residual disease in patients with acute lymphoblastic leukemia.

REFERENCES

1. Owen MJ, Lamb JR, eds. Immune recognition. Oxford: IRL Press, 1988.
2. Marrack P, Kappler J. The T cell receptor. Science 1987;238:1073-9.
3. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
4. Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575-81.
5. Alt, FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. Science 1987;238:1079-87.
6. Royer HD, Reinherz EL. T lymphocytes: ontogeny, function, and relevance to clinical disorders. N Engl J Med 1987;317:1136-41.
7. Strominger JL. Developmental biology of T cell receptors. Science 1989;244:943-50.
8. Cooper MD. B lymphocytes; Normal development and function. N Engl J Med 1987;317:1452-6.
9. Fowlkes BJ, Pardoll DM. Molecular and cellular events of T cell development. Adv Immunol 1989;44:207-63.
10. Haynes BF, Denning SM, Singer KH, Kurtzberg J. Ontogeny of T-cell precursors: a model for the initial stages of human T-cell development. Immunol Today 1989;10:87-91.
11. Campana D. The developmental stages of the human T cell receptors: a review. Thymus 1989;13:3-18.
12. Kisielow P, Blüthmann H, Staerz UD, Steinmetz M, Von Boehmer H. Tolerance in T-cell-receptor transgenic mice involves deletion of nonmature $CD4^+8^+$ thymocytes. Nature 1988;333:742-6.
13. Kisielow P, Sia Teh H, Blüthmann H, Von Boehmer H. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature 1988;335:730-3.
14. Scott B, Blüthmann H, Sia Teh H, Von Boehmer H. The generation of mature T cells requires interaction of the $\alpha\beta$ T-cell receptor with major histocompatibility antigens. Nature 1989;338:591-3.
15. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Ann Rev Immunol 1988;6:629-62.
16. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus, in press.

CHAPTER 2

IMMUNOLOGIC MARKER ANALYSIS

CHAPTER 2.1

INTRODUCTION: MICROSCOPIC TECHNIQUES FOR IMMUNOLOGIC MARKER ANALYSIS

J.J.M. van Dongen[1] and W.M. Comans-Bitter[1,2]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
2. Department of Pediatrics, Sophia Children's Hospital, Rotterdam, The Netherlands.

INTRODUCTION

Leukocytes and their malignant counterparts can be recognized on the basis of morphological and cytochemical characteristics (1-4). A more detailed characterization of leukocytes can be obtained by immunologic marker analysis (5,6). Immunologic markers are proteins or glycoproteins, which are located on the cell membrane or intracellularly; their expression is detectable by use of conventional antisera or monoclonal antibodies (McAb). Several techniques can be used to determine the reactivity of antibodies against leukocyte antigens: rosetting techniques and immunofluorescence, immunogold and immunoenzyme labeling techniques (Table 1).

TECHNIQUES FOR THE DETECTION OF IMMUNOLOGIC MARKERS

In rosetting techniques the relevant antibodies or second step reagents are bound to erythrocytes, latex beads or magnetic beads (7-9). Furthermore, sheep red blood cells (SRBC) can directly bind to human T cells via the CD2 antigen (SRBC receptor), which results in the formation of so-called E rosettes (10,11). A subpopulation of human B lymphocytes can form rosettes with mouse erythrocytes (12,13). Rosetting techniques are generally not used for routine immunophenotyping, but represent easy techniques for cell separation (9,10,14-16).

In immunoenzyme techniques the antibodies are labeled with an enzyme, such as peroxidase (PO), alkaline phosphatase (AP) or β-galactosidase (17-19). These techniques are generally used for immunologic marker analysis of cells in tissue sections. Many of the available anti-leukocyte McAb can be used on acetone fixed frozen sections, but only a few of them are suitable for application on formaldehyde-fixed paraffin-embedded tissue sections (20).

Immunogold techniques in which antibodies are labeled with small colloidal gold particles were first used in electron microscopy, because of the high electron density of the gold particles (21). However, immunogold labeling can also be visualized in light microscopy by use of the same epi-illumination system as in fluorescence microscopy (see below) (21). The immu-

TABLE 1. Microscopic techniques for the detection of immunologic markers.

1.	**Rosette techniques**
	e.g. - E rosette (binding of SRBC to CD2 molecules on T cells)
	- M rosette (binding of mouse erythrocytes to particular receptors on a subpopulation of B lymphocytes)
	- erythrocytes or beads coated with McAb (e.g. magnetic beads for cell separation)
2.	**Immunofluorescence techniques**
	fluorochromes: - fluorescein isothiocyanate (FITC)
	- tetramethylrhodamine isothiocyanate (TRITC)
	- Texas red (TX)
3.	**Immunogold techniques**
	with or without silver enhancement
4.	**Immunoenzyme techniques**
	enzymes, e.g.: - peroxidase (PO)
	- alkaline phosphatase (AP)
	- β-galactosidase

nogold signal can be improved by the so-called silver enhancement (22). Interestingly, this immunogold-silver-staining (IGSS) technique can be combined with routine morphological or cytochemical stainings, because the silver enhancement procedure can be followed by morphological stainings such as May-Grünwald Giemsa or cytochemical stainings such as acid phosphatase or α-naphtylesterase (22,23).

Immunofluorescence (IF) techniques are widely used for immunophenotyping, because they can easily be applicated for both single and double stainings (24). Even triple immunologic stainings can be performed (25-28). The first or second step reagents are conjugated with a fluorochrome as label. In fluorescence microscopy generally fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) or Texas red (TX) are used, whereas FITC and phycoerythrin are used in fluorescence activated flowcytometry (27). For triple immunologic stainings immunogold can be used as third label in fluorescence microscopy (25,26) and allophycocyanin (APC) as third label in flowcytometry (27,28). The possibilities and limitations of flowcytometric analysis of the expression of leukocyte antigens is extensively discussed in several recent publications (29-31). Therefore this Chapter will focus on fluorescence microscopy. Special attention will be paid to detection of intracellular markers and the use of double IF stainings.

IMMUNOFLUORESCENCE MICROSCOPY

For evaluation of immunofluorescence stainings we routinely use Zeiss Standard 16 microscopes (Carl Zeiss, Oberkochen, FRG), equipped with phase contrast facilities and a IV FL Fluoreszenz-Auflichtkondensor (epi-illumination condensor) with a 50 Watt HBO mercury lamp (Osram, Berlin, FRG). These microscopes contain at least two Zeiss immersion-oil objec-

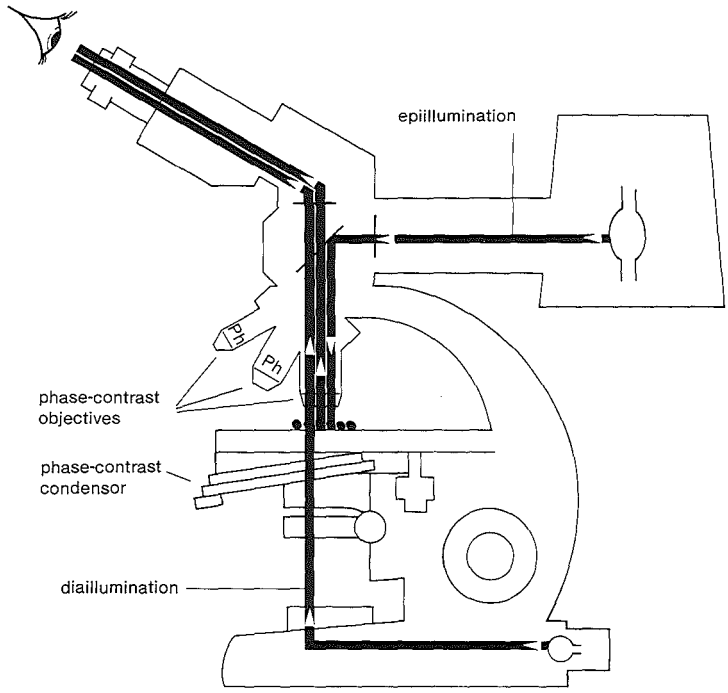


Figure 1. Fluorescence microscope equipped with epiillumination system, phase contrast condensor and phase-contrast objectives.

tives with phase contrast rings: a neofluar 63/1.25 Oel Ph3 for evaluation of the majority of membrane stainings and intracellular stainings and a Planapo 40/1.0 Oel Ph3 for evaluation of strong fluorescence staining patterns such as cytoplasmic immunoglobulin (CyIg) staining in plasma cells. These objectives are used in combination with Zeiss wide-field oculars Kpl-W 10x/18 (Figure 1).

The filter combinations in the epi-illumination system consist of excitation filters, emission filters and a dichroic mirror, which reflects the excitation light but allows the emission light to pass (24). Two filter combinations are used in our fluorescence microscopes: Zeiss filter combination 19 (BP 485/20; FT510; LP515) combined with KP560 filters for the evaluation of FITC labeling and Zeiss filter combination 14 (BP510-560; FT580; LP590) for the evaluation of TRITC labeling (Figure 2).

From our experience phase contrast facilities are essential for optimal evaluation of immunofluorescence stainings, since they provide valuable information about the morphology of the cells and the precise location of the fluorescence labeling. The latter is especially important in case of intracellular markers, since they generally exhibit specific staining patterns, which are dependent on the subcellular location of those markers. An example is given in Figure 3, which illustrates the typical cytoplasmic CD3 (CyCD3) staining in immature T cells (32,33). Detailed information concerning CyCD3 expression is given in Chapter 3.2.

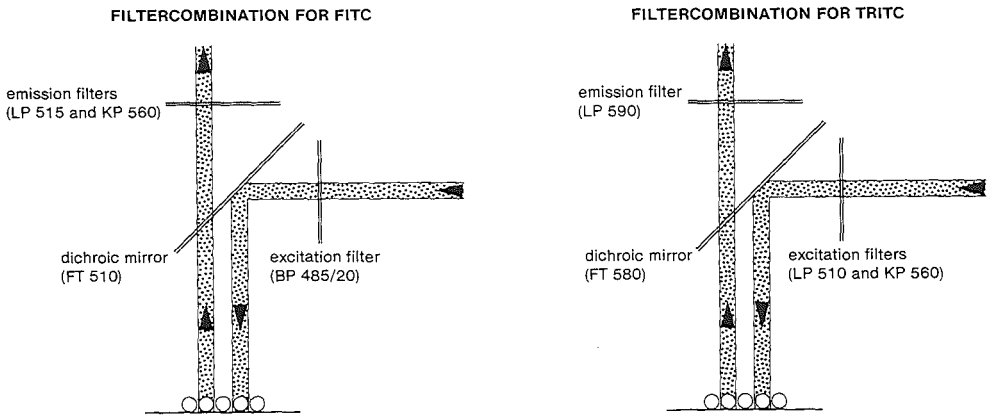


Figure 2. Filter combinations for the visualization of labelings with the fluorochromes FITC and TRITC.

SURFACE MEMBRANE AND INTRACELLULAR IMMUNOLOGIC MARKERS

The majority of the regularly used immunologic markers are surface membrane proteins, which are easily detectable by immunofluorescence labeling of viable cells in suspension and subsequent analysis with a flowcytometer or a fluorescence microscope. Flowcytometric analysis of such immunofluorescence stainings allows the screening of many cells in a short time period (500-5000 per sec) as well as a precise determination of the fluorescence intensity of each cell, which is a measure for the antigen density on the cell surface. Restricted information about size and morphology of the cells can be obtained via forward light scatter (FLS) and perpendicular light scatter (PLS). Also the computerized data analysis and data storage is an advantage of flowcytometry. In fluorescence microscopy only limited numbers of cells can be evaluated per time span and the quantification of fluorescence intensity is a subjective determination. However, detailed information about staining patterns and morphology can easily be obtained via the phase contrast facilities of a fluorescence microscope. The high specificity of the fluorescence microscope allows the detection of a small cell population (< 0.1%) within a large heterogeneous cell sample. Despite these differences, the results of flowcytometric and microscopic analyses of membrane bound immunologic markers are comparable.

However, some important immunologic markers for leukocyte typing are localized intracellularly. Permeabilization of the cell membrane is needed to permit detection of these markers by use of antibodies and information about the precise intracellular localization of the staining is important for correct interpretation. It is obvious that such information can only be obtained by use of a fluorescence microscope and not by use of the current generation of flowcytometers.

Microscopic detection of intracellular markers can be achieved by making cytocentrifuge preparations of the cells under study. These cytocentrifuge preparations are air-dried, fixed with an appropriate fixative (acetone, formol-acetone, acidic ethanol or methanol, depending on the

TABLE 2. Detection of intracellular immunologic markers.

Immunologic marker	Expression pattern (hematopoietic malignancies)	Fixation procedure	Antisera/McAb (isotype)
TdT	- immature lymphoid cells (virtually all ALL and most lymphoblastic lymphomas) - small subpopulation of immature myeloid cells (part of AML)	methanol (30 min, 4°C)	rabbit anti-TdT antisera[a]/ McAb: HTdT-1(γ1)[a], HTdT-3(γ2a)[a], HTdT-4(γ2a)[a]
CyCD3	all immature T cells (all $SmCD3^-$ T-ALL and most $SmCD3^+$ T-ALL)	acetone (10 min, 4°C) or acidic ethanol (15 min, -20°C)	Leu-4 (γ1)[b], UCHT1 (γ1)[c]
CyCD22	immature and mature B cells (majority of precursor-B-ALL)	acetone (10 min, 4°C)	Leu-14 (γ2b)[b], RFB4 (γ)[d], HD39 (γ1)[d]
weak Cyμ	pre-B-cells (pre-B-ALL)	acidic ethanol (15 min, -20°C)	selected anti-μ antisera
CyIg	plasma cells and their precursors (plasmacytoma, immunocytoma, some immunoblastic lymphomas)	acidic ethanol (15 min, -20°C)	anti-Ig antisera
MPO	immature and mature myeloid cells (majority of AML)	acetone (10 min, 4°C)	MPO-7(γ1)[c],CLB-MPO-1(γ2a)[e]
Ki67 antigen	all proliferating cells in late G1, S, G2 and M phases of cell cycle	methanol (30 min, 4°C)	Ki67(γ1)[c]
BrdU incorporation[f]	proliferating cells in S phase of cell cycle	methanol (30 min, 4°C)	anti-BrdU(γ2a) + nuclease[g]

a. Supertechs, Bethesda, MD.
b. Becton Dickinson, San Jose, CA.
c. DAKOPATTS, Glostrup, Denmark.
d. Sera Lab, Crawley Down, UK.
e. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands.
f. The BrdU incorporation assay can only be performed on freshly isolated cells by preincubation with 100 μM BrdU (Sigma, St. Louis, MO) at 37°C for one hour.
g. For optimal detectability of the incorporated BrdU the cytocentrifuge preparations are incubated with a mixture of anti-BrdU McAb and nuclease (Amersham, Amersham, UK) for one hour.

type of immunologic marker), rehydrated by washing in phosphate buffered saline and subsequently incubated with the relevant antibodies. Several important intracellular markers,the appropriate fixation procedures and the relevant antibodies are summarized in Table 2.

The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed in the nucleus of immature lymphoid cells, i.e. precursor B cells in bone marrow and cortical thymocytes (34-36). Also a small subpopulation of immature myeloid cells may express TdT (37).

CyCD3 is found in all immature T cells: in more than 95% of thymocytes and all immature

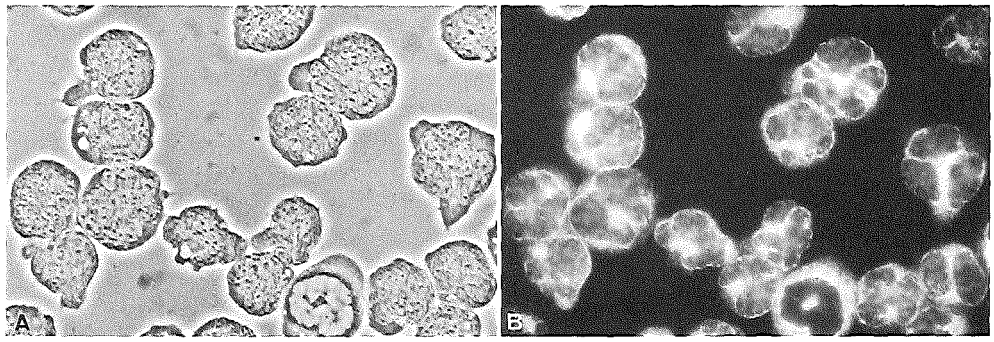


Figure 3. CyCD3 staining on the T cell line Molt4. **A**: phase contrast morphology. **B**: CyCD3$^+$ cells (FITC labeling). This figure illustrates the typical cytoplasmic staining for CD3. The cytoplasmic location of the staining is especially prominent in the mitotic cell.

T cell acute lymphoblastic leukemias (T-ALL) which do not express CD3 on their surface membrane (SmCD3$^-$)(32,33).

The expression of the CD22 antigen on the cell membrane of immature B cells is often weak and it may be difficult to detect by CD22 antibodies in fluorescence microscopy. In cytocentrifuge preparations CD22 expression by precursor B cells is better detectable, which has led to the suggestion that the expression of CD22 antigens in precursor B cells is mainly localized in the cytoplasm (CyCD22) (38). However the CyCD22 staining pattern is not comparable to other typical cytoplasmic staining patterns, but resembles membrane stainings. Evidence for the membrane localization of the CD22 antigen in most precursor B cells has also been found by flowcytometry (39). The improved detectability of CD22 antigens in cytocentrifuge preparations is probably due to a better availability of the antigenic epitopes in fixed cells on cytocentrifuge preparations as compared to viable cells in suspension.

A subset of precursor B cells in bone marrow weakly expresses cytoplasmic μ heavy chains (weak Cyμ), without expression of Ig light chains. These cells have been defined as pre-B cells (40). Strong CyIg expression of both Ig heavy and Ig light chains is found in plasma cells and their precursors (41).

Myeloperoxidase (MPO) is localized in the azurophilic granules of myeloid cells (42). Synthesis of MPO already occurs in early stages of myeloid differentiation (43,44). Most probably MPO is only expressed in myeloid cells and their malignant counterparts, the acute myeloid leukemias (AML) (45). Therefore MPO is an excellent marker for immature myeloid cells and AML (45).

Proliferative activity of cells can be studied by analyzing cycle-associated features such as Ki67 expression (46,47) and 5-bromo-2'-deoxyuridine (BrdU) incorporation (47,48). The nuclear protein Ki67 is expressed during late G1, S, G2 and M phases of the cell cycle and is detectable by use of an antibody (26,46). The incorporation of BrdU in DNA can be detected by use of anti-BrdU antibodies and gives an indication of the percentage of cells in S phase of the cell cycle (26,47,48).

Because of the advantages of speed and quantifiability of fluorescence intensity in case of flowcytometry, several investigators have tried to develop fixation and labeling techniques for

the detection of intracellular markers in cell suspensions (49-51). So far, intracellular background staining and fixation-associated changes in cell size and morphology hamper the flowcytometric detection of intracellular markers. In addition, the flowcytometer cannot provide information about staining patterns and the subcellular location of the staining.

APPLICATION OF DOUBLE IMMUNOFLUORESCENCE STAINING TECHNIQUES

IF techniques are optimally suitable for the simultaneous detection of two different molecules or epitopes. Detailed technical information about the double IF stainings needed for this purpose is given in Chapter 2.3. Double IF stainings are important for both diagnostic and research purposes (Table 3).

Applications of double IF staining techniques in the immunodiagnosis of hematopoietic malignancies are the following: detection of small populations of malignant B cells (>2%) based

TABLE 3. Applications of double IF staining techniques.

A. Immunodiagnosis of hematopoietic malignancies

1. Detection of small populations of malignant Ig^+ B cells (>2%)
   e.g. - careful determination of κ/λ ratio by use of κ/λ double IF stainings or by use of κ/B cell marker and λ/B cell marker double IF stainings

2. Characterization of small malignant cell populations
   e.g. - analysis of TdT^+ cell populations (>1%) in blood or lymph nodes

3. Detection of subpopulations within a malignancy
   e.g. - AML
   - chronic myeloid leukemia in blast crisis

4. Characterization of two hematopoietic malignancies in one patient

5. Detection of minimal residual disease in patients with acute leukemia
   e.g. - TdT^+ T cell malignancies
   - TdT^+ AML

B. Applications in research

1. Determination of the specificity of antibodies
   e.g. - cocapping experiments to study whether particular antibodies recognize the same molecule.

2. Studies on the reactivity patterns of antibodies in normal heterogeneous cell populations

3. Detailed characterization of a particular cell population
   e.g. - analysis of TdT^+ cells in bone marrow
   - analysis of TcR-$\gamma\delta^+$ cells in blood or thymus

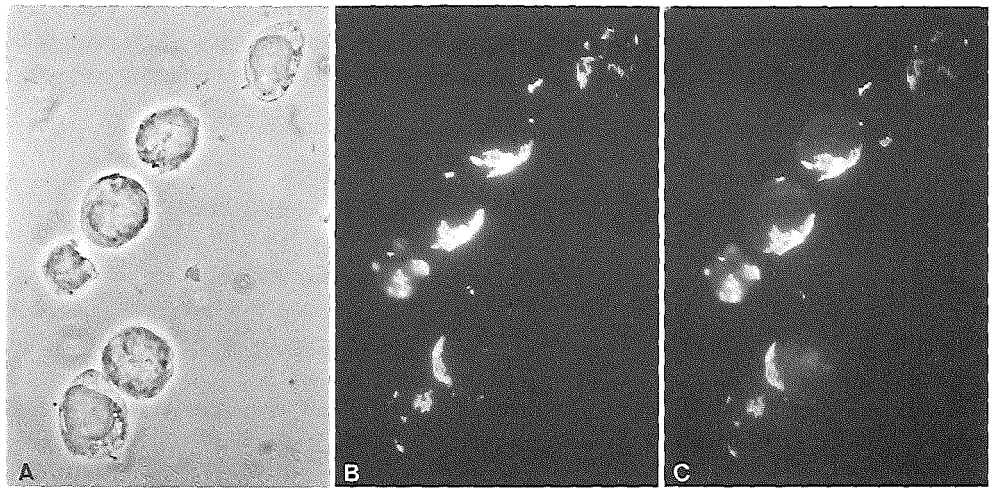


Figure 4. Cocapping experiment using the B cell-specific antibody L26 in conjunction with the CD20 antibody B1 on the B cell line ROS-17. Viable cells were first incubated with the B1 antibody and a TRITC-conjugated second step reagent. These incubations were performed for more than 1 hour at 37°C to enable capping. Cytocentrifuge preparations of the labeled cells were air dried, fixed in acetone (10 min; 4°C) and incubated with biotinylated L26 and FITC-conjugated streptavidin. **A**: phase contrast morphology of the labeled cells; **B**: the same cells showing that CD20 antigen is present in a capped dot-like distribution, often localized at one pole of the cell (TRITC labeling); **C**: L26 staining (FITC labeling) is completely identical in distribution to the CD20 antigen. This figure illustrates that L26 most probably recognizes the CD20 antigen.

on Ig light chain restriction (52,53), immunophenotypic analysis of a small malignant cell population, detection of subpopulations within a malignancy (37,54), and the characterizationof two different hematopoietic malignancies in one patient (55). In our department we especially use double IF staining techniques for detection of minimal residual disease in patients with a TdT^+ T cell malignancy or a TdT^+ AML by use of T cell marker/TdT or myeloid marker/TdT double IF stainings, respectively (56,57). The detection limit of these double staining techniques is extremely low (0.01 - 0.001%), and therefore allows a careful evaluation of the efficacy of the treatment during follow-up of the leukemia patients as well as an early detection of a possible relapse (56). More details about the detection of minimal residual disease by immunologic marker analysis are given in Chapter 5.3 and 5.4.

In research, double IF stainings are useful for studying the specificity of antibodies and for evaluation of their reactivity patterns in normal heterogeneous cell populations. An elegant example is the application of cocapping experiments to determine whether different McAb recognize the same molecule. Using this approach we were able to demonstrate that the B cell specific antibody L26 recognizes an intracellular domain of the CD20 molecule (Figure 4) (58). Finally, double IF stainings are necessary for detailed characterization of small cell populations, such as TdT^+ cells in bone marrow (35-37) or T cell receptor-$\gamma\delta$ $(TcR\text{-}\gamma\delta)^+$ cells in blood or thymus (59,60).

CONCLUSION

Immunofluorescence stainings are important for immunophenotyping of hematopoietic cells and their malignant counterparts as well as for research topics. Especially the easy workability of double IF stainings makes this technique valuable.

Flowcytometric analysis of fluorescence stainings has the advantages of speed and quantifiability of fluorescence intensity. Fluorescence microscopy, on the other hand, has the advantages of high specificity and high sensitivity, especially in case of intracellular immunologic markers.

REFERENCES

1. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. Br J Haematol 1976;33:451-8.
2. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7): a report of the French-American-British cooperative group. Ann Intern Med 1985;103:460-2.
3. Bennett JM, Catovsky D, Daniel MT, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposed revised criteria for the classification of acute myeloid leukemia: a report of the French-American-British cooperative group. Ann Intern Med 1985;103:626-9.
4. The non-Hodgkin's lymphoma pathologic classification project. National cancer institute sponsored study of classifications of Non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. Cancer 1982;49:2112-35.
5. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
6. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33:298-314.
7. Ling NR, Richardson PR. A critical appraisal of the direct antibody-rosette test for the detection of cell surface antigens. J Immunol Methods 1981;47:265-74.
8. Mirro J, Schwartz JF, Civin CI. Simultaneous analysis of cell surface antigens and cell morphology using monoclonal antibodies conjugated to fluorescent microspheres. J Immunol Methods 1981;47:39-48.
9. Kemshead JT, Ugelstad J. Magnetic separation techniques: their application to medicine. Mol Cell Biochem 1985;67:11-8.
10. Elliott BE. A sensitive method for the separation of rosette-forming cells. In: Lefkovits I, Pernis B, eds. Immunological methods, Academic Press Inc, 1979:241-59.
11. Moore AL, Zusman J. Conditions enhancing the percentage and size of E rosettes formed by T lymphocytes. J Immunol Methods 1978;23:275-84.
12. McGraw DJ, Kurec AS, Davey FR. Mouse erythrocyte formation. A marker for resting B lymphocytes. Am J Clin Pathol 1981;77:177-83.
13. Caligaris-Cappio F, Gobbi M, Bofill M, Janossy G. Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukemia. J Exp Med 1982;155:623-8.
14. Falkoff RM, Peters M, Fauci AS. T cell enrichment and depletion of human peripheral blood mononuclear cell preparations. Unexpected findings in the study of the functional activities of the separated populations. J Immunol Methods 1982;50:39-49.
15. Vartdal F, Kvalheim G, Lea TE, Bosnes V, Gaudernack G, Ugelstad J, Albrechtsen D. Depletion of T lymphocytes from human bone marrow: use of magnetic monosized polymer microspheres coated with T-lymphocyte-specific monoclonal antibodies. Transplant 1987;43:366-371.
16. Abts H, Emmerich M, Miltenyi S, Radbruch A, Tesch H. CD20 positive human B lymphocytes separated with the magnetic cell sorter (MACS) can be induced to proliferation and antibody secretion in vitro. J Immunol Methods 1989;125:19-28.

17. Mason DY, Sammons R. Alkaline phosphatase and peroxidase for double immunoenzymatic labelling of cellular constituents. J Clin Pathol 1978;31:454-60.
18. Cordell JL, Falini B, Erber WN, Ghosh AK, Abdulaziz Z, MacDonald S, Pulford KAF, Stein H, Mason DY. Immunoenzymatic labeling of monoclonal antibodies using immune complexes of alkaline phosphatase and monoclonal anti-alkaline phosphatase (APAAP complexes). J Histochem Cytochem 1984;32:219-29.
19. Leenen PJ, Melis ML, Van Ewijk W. Single-cell immuno-β-galactosidase staining of heterogeneous populations. Practical application on limited cell numbers. Histochem J 1987;19:497-503.
20. Norton AJ, Isaacson PG. Lymphoma phenotyping in formalin-fixed and paraffin wax-embedded tissues. I. Range of antibodies and staining patterns. Histopathol 1989;14:437-46.
21. De Mey J. Colloidal gold probes in immunocytochemistry. In: Polak JM, Van Noorden S, eds. Immunocytochemistry, Practical Applications in Pathology and Biology. Boston: Wright PSG, 1983;1:82-112.
22. De Waele M, De Mey J, Renmans W, Labeur C, Reynaert Ph, Van Camp B. An immunogold-silver staining method for detection of cell-surface antigens in light microscopy. J Histochem Cytochem 1986;34:935-9.
23. De Waele M, De Mey J, Renmans W, Labeur C, Jochmans K, Van Camp B. Potential of immunogold-silver staining for the study of leukocyte subpopulations as defined by monoclonal antibodies. J Histochem Cytochem 1986;34:1257-63.
24. Hijmans W, Haaijman JJ, Schuit HRE. Immunofluorescence. In: Adler WM, Nordin AA, eds. Immunological techniques applied to aging research. Boca Raton: CRC Press Inc, 1981:141-63.
25. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J. Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.
26. Campana D, Coustan-Smith E, Janossy G. Double and triple staining methods for studying the proliferative activity of human B and T lymphoid cells. J Immunol Methods 1988;107:79-88.
27. Coon JS, Landay AL, Weinstein RS. Biology of disease. Advances in flow cytometry for diagnostic pathology. Lab Invest 1987;57:453-79.
28. Loken MR, Keij JF, Kelley KA. Comparison of helium-neon and dye lasers for the excitation of allophycocyanin. Cytometry 1987;8:96-100.
29. Keren DF, ed. Flow cytometry in clinical diagnosis. Chicago: ASCP Press, 1989.
30. Adriaansen HJ, Van Dongen JJM, eds. Klinische toepassingen van flowcytometrie. Rotterdam: Department of Immunology of the Erasmus University, 1990.
31. Laeren OD, Bjerkners R. Flowcytometry in hematology. Analytical cytology series. Londen: Academic press, 1990, in press.
32. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
33. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
34. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
35. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
36. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
37. Adriaansen HJ, Hooijkaas H, Kappers-Klunne MC, Hählen K, Van't Veer MB, Van Dongen JJM. Double marker analysis for terminal deoxynucleotidyl transferase and myeloid antigens in acute nonlymphocytic leukemia patients and healthy subjects. Haematol Blood Transf 1990;33:41-9.
38. Campana D, Janossy G, Bofill M, Trejdosiewicz LK, Ma D, Hoffbrand V, Mason DY, LeBacq A-M, Forster HK. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985;134:1524-30.
39. Boué DR, LeBien TW. Expression and structure of CD22 in acute leukemia. Blood 1988;71:1480-6.
40. Gathings WE, Lawton AR, Cooper MD. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur J Immunol 1977;7:804-10.
41. Hijmans W, Schuit HRE, Klein F. An immunofluorescence procedure for the detection of intracellular

immunoglobulins. Clin exp Immunol 1969;4:457-72.
42. Ford Bainton D, Ullyot JL, Farquhar MG. The development of neutrophilic polymorphonuclear leukocytes in human bone marrow: origin and content of azurophil and specific granules. J Exp Med 1971;134:907-34.
43. Yamada M. Myeloperoxidase precursors in human myeloid leukemia HL-60 cells. J Biol Chem 1982;257:5980-2
44. Nauseef WM, Olsson I, Arnljots K. Biosythesis and processing of myeloperoxidase - A marker for myeloid cell differentiation. Eur J Haematol 1988;40:97-110.
45. Van der Schoot CE, Daams GM, Pinkster J, Vet R, Von dem Borne AEGK. Monoclonal antibodies against myeloperoxidase are valuable immunological reagents for the diagnosis of acute myeloid leukaemia. Br J Haematol 1990;74:173-8.
46. Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H. Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol 1984;133:1710-5.
47. Campana D, Janossy G. Proliferation of normal and malignant human immature lymphoid cells. Blood 1988;71:1201-10.
48. Gratzner HG. Monoclonal antibody to 5-Bromo- and 5-Iododeoxyuridine: a new reagent for detection of DNA replication. Science 1982;218:474-5.
49. Mirro J Jr, Kitchingman G, Behm FG, Murphy SB, Goorha RM. T cell differentiation stages identified by molecular and immunologic analysis of the T cell receptor complex in childhood lymphoblastic leukemia. Blood 1987;69:908-12.
50. Slaper-Cortenbach ICM, Admiraal LG, Kerr JM, Van Leeuwen EF, Von dem Borne AEGKr, Tetteroo PAT. Flow-cytometric detection of terminal deoxynucleotidyl transferase and other intracellular antigens in combination with membrane antigens in acute lymphatic leukemias. Blood 1988;72:1639-44.
51. Kastan MB, Slamon DJ, Civin CI. Expression of protooncogene c-*myc* in normal human hematopoietic cells. Blood 1989:73:1444-51.
52. Smith BR, Weinberg DS, Robert NJ, Towle M, Luther E, Pinkus GS, Ault KA. Circulating monoclonal B lymphocytes in non-Hodgkin's lymphoma. N Engl J Med 1984;311:1476-81.
53. Nakano M, Kuge S, Kuwabara S, Yaguchi M, Kawanishi Y, Kodama K, Akiya T, Toyama K. The basic study on κ-λ imaging by δ-curve for the detection of a monoclonal B-cell population in the peripheral blood. Blood 1988;72:1461-6.
54. Van Dongen JJM, Hooijkaas H, Hagemeijer A, Hählen K, Van Zanen GE, Lansdorp PM. Characterization and monitoring of the malignant cells in the blastic and chronic phase of chronic myeloid leukemia. In: Peeters H ed. Protides of the biological fluids. Oxford: Pergamon Press, 1984;31:711-4.
55. Hagemeijer A, Bolhuis RLH, Van Dongen JJM, Sizoo W. Simultaneous occurrence of acute monoblastic leukemia and an abnormal B-cell clone with both cell types characterized by specific cytogenetic and immunological markers. Scand J Haematol 1986;36:484-91.
56. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986:113-33.
57. Adriaansen HJ, Van Dongen JJM, Kappers-Klunne MC, Hählen K, Van't Veer MB, Wijdenes-de Bresser JHFM, Holdrinet ACJM, Harthoorn-Lasthuizen EJ, Abels J, Hooijkaas H. Terminal deoxynucleotidyl transferase positive subpopulations occur in the majority of ANLL: implications for the detection of minimal disease. Leukemia 1990, in press.
58. Mason DY, Comans-Bitter WM, Cordell JL, Verhoeven M-AJ, Van Dongen JJM. Antibody L26 recognizes an intracellular epitope on the B cell associated CD20 antigen. Am J Pathol 1990, in press.
59. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.
60. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.

CHAPTER 2.2

IMMUNOLOGIC MARKERS AND THEIR CD CODES

Jacques J.M. van Dongen, Henk J. Adriaansen and Herbert Hooijkaas

Department of Immunology, Erasmus University/ University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

For the detection of immunologic markers on hematopoietic cells generally monoclonal antibodies (McAb) are used. During the last ten years many McAb against immunologic markers of leukocytes have become available. To create clearness and order within the large panels of McAb an international nomenclature has been designed (1-4).

During four Leucocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989) a large part of the McAb against leukocyte antigens have been grouped into antibody clusters based on their reactivity with identical antigens (Table 1). Each cluster has its own code, the so-called CD ("cluster of differentiation" or "cluster designation") code (1-4). In this way McAb against e.g. the T cell markers T6 and T11 have received the codes CD1 and CD2, respectively, while the CD3 code is assigned to McAb against the T cell receptor (TcR) associated CD3 chains.

CD NOMENCLATURE

In principle CD codes are only assigned, if three or more McAb from different laboratories recognize the same antigen, of which the molecular (mol.) mass as well as the expression pattern has been determined. However, in a few clusters only two antibodies are present; in some other clusters antibodies of only two laboratories are available and sometimes information about the recognized immunologic marker is lacking (e.g. the mol. mass). In such cases the CD code is supplied with the letter "w" (workshop), which indicates that it concerns a preliminary clustering.

The name of the McAb, which has been used to detect a specific CD molecule, can be placed in parentheses behind the CD code. In this way, code CD3 can be extended to CD3(OKT3), CD3(Leu-4), CD3(VIT-3) or CD3(UCHT1), depending on the antibody used. Such a supplementation is important for optimal comparison of data from different laboratories, since McAb of the same cluster may slightly differ in their reaction pattern. For instance, the majority

TABLE 1. CD codes as established during the four Leucocyte Typing Conferences.

Leucocyte Typing Conference		Number of submitted monoclonal antibodies	Number of established CD codes
I	Paris, 1982	~ 150	CD1 - CDw15
II	Boston, 1984	~ 350	CD16 - CDw26
III	Oxford, 1986	~ 900	CD27 - CD45
IV	Vienna, 1989	~ 1100	CD46 - CDw78

of CD3 antibodies are able to recognize CD3-ϵ chains at the cell surface of viable cells (5), but cytoplasmic CD3 chains (CyCD3) in fixed cytocentrifuge preparations or frozen sections can be recognized by only a few CD3 antibodies (6). Apparently not all epitopes on the CD3-ϵ chain are resistant to denaturation by acetone or ethanol fixation of cyto-centrifuge preparations or frozen sections.

So far 78 CD codes have been assigned. A condensed summary of these clusters, the antigen recognized and the reactivity pattern of the clustered antibodies is given in Table 2. Several clusters are subdivided with letter codes, because the antibodies of these clusters recognize antigens which are homologous, but not identical (e.g. CD1 and CD11 antigens) (7-10). The genes which code for these molecules belong to the same gene family (7-10). In other clusters the subdivision is based on reactivity of the antibodies with different isoforms of a CD molecule, which represent different splicing products from the same gene complex (e.g. CD45 molecules) (11,12). However, antibodies against complement receptor type 2 (CR2), complement receptor type 1 (CR1) and the decay-accelerating factor were grouped into different clusters (CD21, CD35 and CD55, respectively), although they belong to the same gene cluster of regulators of complement activation (13).

A detailed summary of all CD clusters is given in the Appendix of this Chapter. This Appendix summarizes relevant information about the various McAb (isotype, origin, reactivity pattern with hematopoietic cells) as well as the recognized immunologic markers (alternative name, known function, mol. mass, etc.). Most antibodies mentioned in the Appendix are commercially available (see footnotes of the Appendix).

FUNCTIONS OF CD MOLECULES

During the first three Leucocyte Typing Conference only limited information about functions of CD molecules was available. However, during the fourth conference in Vienna in 1989 more functional data were presented. Several CD molecules appear to play a role in signal transduction and activation of B lymphocytes (e.g. CD19, CD20 and CD22 molecules) or T lymphocytes (e.g. CD2, CD3 and CD5 molecules)(4), while other CD molecules exhibit receptor functions (1-4,14,15), are involved in cell-cell interactions (10,16-18) or represent homing receptors (19-22).

TABLE 2. CD nomenclature as established during the four Leucocyte Typing Conferences.

CD code	CD antigen	Reactivity	CD code	CD antigen	Reactivity	CD code	CD antigen	Reactivity
CD1	T6 antigen	Thy, sub-B, DC	CD28	Tp44 antigen	sub-T	CD51	VNR-α chain	Plt
CD2	T2 antigen, LFA-2	pan-T	CD29	integrin β1 chain	broad	CDw52	Campath-1	broad
CD3	T3 antigen	T	CD30	Ki-1 antigen	act-T, act-B, RS	CD53	gp32-40 antigen	leukocytes
CD4	T4 antigen	sub-T	CD31	platelet GPIIa'	Plt, Mo, G, sub-T	CD54	ICAM-1	broad
CD5	T1 antigen	T	CDw32	FcγRII	Mo, G, B, Eo	CD55	DAF	broad
CD6	T12 antigen	T	CD33	gp67 antigen	My, Mo	CD56	NCAM	sub-T, NK
CD7	Tp41 antigen	pan-T	CD34	gp115 antigen	prog-leukocytes	CD57	HNK1	sub-T, sub-NK
CD8	T8 antigen	sub-T	CD35	CR1	B, G, Mo	CD58	LFA-3	broad
CD9	p24 antigen	sub-B, Plt	CD36	platelet GPIV	Plt, prog-Ery, Mo	CD59	Ly-6 antigen	broad
CD10	CALLA	prog-B	CD37	B cell antigen	B	CDw60	NeuAc-NeuAc-Gal	sub-T, Plt
CD11a	LFA-1	Ly, My, Mo	CD38	T10 antigen	prog-Ly, act-T, PC	CD61	integrin β3 chain	Plt
CD11b	MAC-1, CR3	My, Mo	CD39	gp80 antigen	B	CD62	GMP 140	act-Plt
CD11c	p150,95	My, Mo, sub-B	CD40	gp50 antigen	sub-B, DC	CD63	GP-53	act-Plt, Mo, G
CDw12	myeloid antigen	My, Mo, Plt	CD41	platelet GPIIb-IIIa	Plt	CD64	FcγRI	Mo
CD13	aminopeptidase N	My, Mo	CD42a	platelet GPIX	Plt	CDw65	fucoganglioside	My, Mo
CD14	gp55 antigen	Mo	CD42b	platelet GPIb	Plt	CD66	gp180-200 antigen	G
CD15	X hapten, FAL	My, G	CD43	leukosialin	broad	CD67	p100 antigen	G
CD16	FcγRIII	NK, G	CD44	Pgp-1, homing-R	broad	CD68	macrophage antigen	Mϕ
CDw17	lactosylceramide	Mo, G, Plt	CD45	LCA	leukocytes	CD69	AIM	act-T, act-B
CD18	integrin β2 chain	Ly, My, Mo	CD45RO	restricted LCA	sub-T, G, Mo	CDw70	Ki-24 antigen	act-T, act-B, RS
CD19	gp90 antigen	pan-B	CD45RA	restricted LCA	sub-T, B, G, Mo	CD71	transferrin receptor	act-leukocytes
CD20	p35 antigen	B	CD45RB	restricted LCA	sub-T, B, G, Mo	CD72	gp43/39 antigen	pan-B
CD21	CR2, EBV-R	sub-B	CD46	MCP	broad	CD73	ecto-5'-NT	B, sub-T
CD22	gp135 antigen	pan-B	CD47	gp47-52 antigen	broad	CD74	invariant chain	B, sub-Mo
CD23	FcεRII	B, Mo, Eo	CD48	gp41 antigen	leukocytes	CDw75	B cell antigen	sub-B, sub-T
CD24	BA-1 antigen	sub-B, G	CDw49b	VLA-α2	Plt, act-T	CD76	gp85/87 antigen	sub-B, sub-T
CD25	IL-2 receptor	act-T, act-B, act-Mϕ	CDw49d	VLA-α4	B, T, Mo	CD77	Gb3 antigen	act-B
CD26	DPP IV	act-T, act-B, Mϕ	CDw49f	VLA-α6	Plt, sub-T	CDw78	B cell antigen	B, sub-Mϕ
CD27	p110 antigen	sub-T, act-T, sub-B	CDw50	gp148/108 antigen	leukocytes			

Abbreviations used: act = activated, AIM = activation inducer molecule, B = B cells, broad = broad tissue distribution, CALLA = common ALL antigen, CD = cluster of differentiation/cluster designation, CR = complement receptor, DAF = decay accelerating factor, DC = dendritic cells, DPP IV = dipeptidylpeptidase IV, EBV = Epstein-Barr virus, Eo = eosinophils, ery = erythrocytes, FAL = fucosyl-N-acetyl-lactosamin, FcεR = Fc receptor for IgE, FcγR = Fc receptor for IgG, G = granulocytes, GMP = granule membrane protein, GP = glycoprotein, HNK = human natural killer cells, ICAM = intercellular adhesion molecule, IL-2 = interleukin 2, LCA = leukocyte common antigen, LFA = leukocyte function antigen, Ly = lymphocytes, MCP = membrane cofactor proteine, Mϕ = macrophages, Mo = monocytes, My = myeloid cells, NCAM = neural cell adhesion molecule, NeuAc-NeuAc-Gal = disialosyl group, NK = natural killer cells, 5´-NT = 5´-nucleotidase, PC = plasma cells, Plt = platelets, prog = progenitor, R = receptor, RS = Reed Sternberg cells, sub = subpopulation, T = T cells, Thy = thymocytes, VLA = very late activation, VNR = vitronectin receptor.

Antibodies against the three types of Fc receptors for IgG, FcγRI, FcγRII and FcγRIII, have received the codes CD64, CDw32 and CD16, respectively (4,14). Antibodies against the three complement receptors, CR1, CR2 and CR3, are grouped into the clusters CD35, CD21 and CD11b/CD18, respectively (2-4).

During the last three years a new group of intercellular adhesion molecules has been recognized, the so-called integrins (10,17). This family of cell surface receptors consists of at least six subfamilies, based on the use of common β chains, which differ between the subfamilies but have a high degree of homology (10,17). Antibodies against members of three integrin subfamilies have received CD codes. The very late activation (VLA) antigen subfamily consists of at least six different α chains (CDw49 antigens) and one common β1 integrin chain (CD29 antigen)(10,17,18). The leukocyte adhesion molecule (LeuCAM) family consists of three different α chains (CD11 antigens) and a common β2 integrin chain (CD18 antigen)(10), while the cytoadhesin family consists of at least two α chains (GPIIb of the CD41 molecule and CD51) and one common integrin β3 chain (CD61 antigen) (10,17).

Some CD molecules represent homing receptors. CD44 molecules are homing receptors on the cell surface of lymphocytes, allowing selective binding of the lymphocytes to the endothelium of high endothelial venules (HEV) (19-21). The CDw49d/CD29 molecule probably represents one of the homing receptors for Peyer's patches (22).

Interestingly, several CD molecules appear to represent membrane bound enzymes, such as CD10 (neutral endopeptidase), CD13 (aminopeptidase N), CD26 (dipeptidylpeptidase IV) and CD73 (ecto-5´-nucleotidase)(4). The precise role of these membrane bound enzymes in differentiation, activation or other cell functions has still to be determined.

The available data concerning functions of CD molecules indicate that most cell surface molecules are involved in complex cellular interactions or represent receptors for signals which induce activation or proliferation of the cell or lead to changes in the differentiation status of the cell. Future Leucocyte Typing Conferences will probably not only establish new antibody clusters, but will also contribute more information on functions of CD molecules. This will further enhance the progress in our understanding of differentiation, migration, cell-cell interaction, activation and proliferation of both immune and non-immune cells.

REACTIVITY OF CLUSTERED McAb WITH VIABLE AND FIXED CELLS

The majority of clustered McAb can be used for the detection of immunologic markers on the cell surface of viable cells in suspension. Several of these antibodies can also be used on acetone-fixed or ethanol-fixed cytocentrifuge preparations or frozen sections. However, the reactivity with formalin-fixed paraffin-embedded tissue sections is restricted to a small number of clustered McAb (4,23-26).

In most laboratories for Clinical Pathology the majority of tissue samples are routinely processed by formalin fixation and paraffin embedding. Therefore, it is important that these laboratories have access to antibodies which can react with routinely processed tissue sections. Preferably, clustered antibodies should be used, so that immunophenotyping results from routi-

TABLE 3. Clustered McAb that can be used on formaldehyde-fixed, paraffin-embedded tissue sections.

CD code	McAb[a]	Reactivity with hematopoietic cells
CD3	only polyclonal rabbit antiserum available[b]	immature T cells (cytoplasmic expression; CyCD3) and mature T cells (membrane expression; SmCD3)
CD15	Leu-M1(μ)[c], Tü9(μ)[d]	myeloid cells, granulocytes
CD20	L26(γ2a)[b]	B cells, follicular dendritic reticulum cells
CD30	Ber-H2(γ1)[b]	subpopulation of activated lymphocytes, Reed-Sternberg cells
CD35	7E11(γ1)[e]	B cells, granulocytes, monocytes, dendritic reticulum cells
CD43	DFT-1(γ1)[b], Leu-22(γ1)[c]	T cells, monocytes, granulocytes (broad tissue distribution)
CD45RO	UCHL1(γ2a)[b]	thymocytes, activated T cells, memory T cells
CD45RA	4KB5(γ1)[b], MB1(γ1)[d], MT2(γ1)[d]	virgin T cells, B cells, granulocytes, monocytes
CD45RB	PD7/26(γ1)[b]	B cells, subpopulation of T cells, monocytes, macrophages, granulocytes
CD61	Y2/51(γ1)[b]	megakaryocytes, platelets
CD68	KP1(γ1)[b]	macrophages (especially cytoplasmic expression)
CD71	Ber-T9(γ1)[b]	proliferating cells, activated cells
CD74	LN2(γ1)[d]	subpopulation of precursor B cells, B cells, subpopulation of monocytes
CDw75	LN1(μ)[d]	subpopulation of mature B cells (especially germinal center B cells), subpopulation of T cells (weak)
CD76	HD66(μ)[f], CRIS-4(μ)[g]	subpopulation of mature B cells (especially mantle zone B cells), subpopulation of T cells

a. Detailed information on these McAb can be found in references 4, 23-26.
b. DAKOPATTS, Glostrup, Denmark.
c. Becton Dickinson, San Jose, CA.
d. Biotest, Dreieich, FRG.
e. Dr. N.M. Hogg, London, UK.
f. Dr. G. Moldenhauser, Heidelberg, FRG.
g. Dr. R. Vilella, Barcelona, Spain.

nely processed tissue sections can be compared with results from immunophenotyping on frozen sections or cell suspensions. Therefore, we have summarized all available clustered McAb which can be used on formalin-fixed paraffin-embedded tissue sections (Table 3)(4,23-26).

CONCLUSION

The international CD nomenclature has created order and clearness in the abundance of available McAb against leukocyte antigens. This CD nomenclature enables the composition of representative antibody panels, which allow comparison of immunophenotyping results from different laboratories. This is important for designing internationally accepted immunophenotyping protocols for diagnostic purposes.

Not all detailed information concerning the CD nomenclature is easily accessible. For instance, it is not always clear which antibodies have officially been clustered and whether the relevant antibodies are commercially available. In the Appendix of this Chapter, we therefore summarize all relevant information about clustered McAb and several non-clustered antibodies, which are important for immunophenotyping of hematopoietic cells.

ACKNOWLEDGMENTS. We gratefully thank Professor Dr. R. Benner for his continuous support and Mrs. W.M. Comans-Bitter for critically reading the manuscript and the Appendix.

REFERENCES

1. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte Typing. Human leucocyte differentiation antigens detected by monoclonal antibodies. Berlin: Springer-Verlag, 1984.
2. Reinherz EL, Haynes BF, Nadler LM, Bernstein ID, eds. Leucocyte Typing II. Volume 1: Human T lymphocytes; Volume 2: Human B lymphocytes; Volume 3: Human myeloid and hematopoietic cells. Berlin: Springer-Verlag, 1986.
3. McMichael AJ, Beverley PCL, Gilks W, Horton M, Mason DY, Cobbold S, Gotch FM, Ling N, Milstein C, Waldmann H, Crumpton MJ, Hogg N, MacLennan ICM, Spiegelhalter D, eds. Leucocyte Typing III: White cell differentiation antigens. Oxford: Oxford University Press, 1987.
4. Knapp W, Dörken B, Rieber EP, Stein H, Gilks WR, Schmidt RE, Von dem Borne AEGKr, eds. Leucocyte Typing IV: White cell differentiation antigens. Oxford: Oxford University Press, 1989.
5. Transy C, Moingeon PE, Marshall B, Stebbins C, Reinherz EL. Most anti-human CD3 monoclonal antibodies are directed to the CD3 ϵ subunit. Eur J Immunol 1989;19:947-50.
6. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
7. Martin LH, Calabi F, Lefebvre F-A, Bilsland CAG, Milstein C. Structure and expression of the human thymocyte antigens CD1a, CD1b, and CD1c. Proc Natl Acad Sci USA 1987;84:9189-93.
8. Calabi F, Jarvis JM, Martin L, Milstein C. Two classes of CD1 genes. Eur J Immunol 1989;19:285-92.
9. Corbi AL, Larson RS, Kishimoto TK, Springer TA, Morton CC. Chromosomal location of the genes encoding the leukocyte adhesion receptors LFA-1, Mac-1 and p150,95. J Exp Med 1988;167:1597-607.
10. Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood 1990;75:1037-50.
11. Streuli M, Hall LR, Saga Y, Schlossman SF, Saito H. Differential usage of three exons generates at least five different mRNAs encoding human leukocyte common antigens. J Exp Med 1987;166:1548-66.
12. Streuli M, Morimoto C, Schrieber M, Schlossman SF, Saito H. Characterization of CD45 and CD45R monoclonal antibodies using transfected mouse cell lines that express individual human leukocyte common antigens. J Immunol 1988;141:3910-4.
13. Rey-Campos J, Rubinstein P, Rodriguez de Cordoba S. A physical map of the human regulator of complement activation gene cluster linking the complement genes CR1, CR2, DAF and C4BP. J Exp Med 1988;167:664-

9.
14. Hogg N. The structure and function of Fc receptors. Immunol Today 1988;9:185-7.
15. Fanger MW, Shen L, Graziano RF, Guyre PM. Cytotoxicity mediated by human Fc receptors for IgG. Immunol Today 1989;10:92-9.
16. Springer TA, Dustin ML, Kishimoto TK, Marlin SD. The lymphocyte function-associated LFA-1, CD2, and LFA-3 molecules: cell adhesion receptors of the immune system. Ann Rev Immunol 1987;5:223-52.
17. Hynes RO. Integrins: a family of cell surface receptors. Cell 1987;48:549-54.
18. Hemler ME. Adhesive protein receptors on hematopoietic cells. Immunol Today 1988;9:109-13.
19. Stoolman LM. Adhesion molecules controlling lymphocyte migration. Cell 1989;56:907-10.
20. Pals ST, Hogervorst F, Keizer GD, Thepen T, Horst E, Figdor CC. Identification of a widely distributed 90-kDa glycoprotein that is homologous to the hermes-1 human lymphocyte homing receptor. J Immunol 1989;143:851-7.
21. De los Toyos J, Jalkanen S, Butcher EC. Flow cytometric analysis of the Hermes homing-associated antigen on human lymphocyte subsets. Blood 1989;74:751-60.
22. Holzmann B, Weissman IL. Peyer's patch-specific lymphocyte homing receptors consist of a VLA-4-like α chain associated with either of two integrin β chains, one of which is novel. EMBO J 1989;8:1735-41.
23. Poppema S, Hollema H, Visser L, Vos H. Monoclonal antibodies (MT1, MT2, MB1, MB2, MB3) reactive with leukocyte subsets in paraffin-embedded tissue sections. Am J Pathol 1987;127:418-29.
24. Norton AJ, Isaacson PG. Lymphoma phenotyping in formalin-fixed and paraffin wax-embedded tissue: I. Range of antibodies and staining patterns. Histopathol 1989;14:437-46.
25. Norton AJ, Isaacson PG. Lymphoma phenotyping in formalin-fixed and paraffin wax-embedded tissues: II. Profiles or reactivity in the various tumour types. Histopathol 1989;14:557-79.
26. Mason DY, Comans-Bitter WM, Cordell JL, Verhoeven M-AJ, Van Dongen JJM. Antibody L26 recognizes an intracellular epitope on the B cell associated CD20 antigen. Am J Pathol 1990, in press.

APPENDIX. Summary of clustered and non-clustered immunologic markers.

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD1	T6 antigen; common thymocyte antigen/ MHC-like protein; can associate with β2-microglobulin	CD1a:gp49	Leu-6(γ2b)[b], OKT6(γ1)[c,d], T6(γ1)[e], NA1/34(γ2a)[f,g], VIT-6(γ2a)[h]	- cortical thymocytes (strong), Langerhans cells, subpopulation of dendritic cells, subpopulation of B cells
		CD1b:gp45	7C4/160/4G9 (γ1)[i]	- cortical thymocytes (moderate), Langerhans cells, subpopulation of dendritic cells, subpopulation of B cells
		CD1c:gp43	7C6/162/3B10(μ)[i]	- cortical thymocytes (weak), subpopulation of B cells, Langerhans cells, subpopulation of dendritic cells
CD2	T11 antigen; SRBC receptor (=E rosette receptor); LFA-2/ receptor for T cell activation; ligand for CD58 (LFA-3)	gp50	Leu-5b(γ2a)[b], OKT11(γ2a)[c,d], T11(γ1)[e], CLB-T11/1(γ1)[k], TM1(μ)[d], MT910(γ1)[f], MT1110(γ1)[h]	all T cells, most NK cells; three different antigenic epitopes are known, of which one is the SRBC binding site
CD2R	CD2 epitope restricted to activated T lymphocytes	gp50	VIT-13(μ)[i]	activated T lymphocytes
CD3	T3 antigen (associated with TcR)/signal transduction from TcR to cytoplasm	gp16-25	Leu-4/SK7(γ1)[b], OKT3(γ2a)[c,d], T3(γ1)[e], T3-4B5(γ1)[f], SL5(γ3)[g], BW242/55(γ3)[h], VIT-3(μ)[i], CLB-T3/2(γ2a)[k], UCHT1(γ1)[f,l] (most CD3 McAb recognize the CD3-ε chain)	immature T cells (cytoplasmic expression; CyCD3) and mature functional T cells (membrane expression); the CD3 antigen consists of at least five protein chains; NK cells only express CD3-ζ chains
		CD3-γ: gp25	only polyclonal antisera	these antibodies can detect intracellular CD3 chains
		CD3-δ: gp20	SP-64(γ1)[m], SP-78(γ1)[m]	
		CD3-ε: p20	Leu-4/SK7(γ1)[b], VIT-3b(γ1)[i], UCHT1(γ1)[f,l], SP-6(γ1)[m]	
		CD3-ζ:gp16/16	TIA-2(γ1)[n]	
CD4	T4 antigen/involved in MHC-class-II-restricted antigen recognition; HIV receptor	gp60	Leu-3a(γ1)[b], OKT4(γ2b)[c,d], T4A(γ1)[e], T3-10(γ1)[f], BT5.9(γ3)[g], BW264/123(μ)[h], CLB-T4/1(γ2a)[k], TT4(γ2a)[o], T151(γ2a)[p]	subpopulation of cortical thymocytes, helper/inducer T cells, subpopulation of monocytes and macrophages
CD5	T1 antigen/function in T cell proliferation	gp67	Leu-1(γ2a)[b], OKCLL(γ2a)[c], OKT1(γ1)[d], T1B(γ2a)[e], MID5(μ)[g], CLB-T1/1(γ1)[k], DK23(γ2a)[f]	thymocytes and mature T lymphocytes, subpopulation of B cells (e.g. B-CLL)
CD6	T12 antigen; pan-T cell antigen	gp120	OKT17(γ2)[c], T12(μ)[e], ST23(γ2a)[f], Tp120(γ2a)[g], VIT-12(γ1)[i], Tü33(γ2a)[o], T411(γ1)[p]	thymocytes and mature T lymphocytes, subpopulation of B cells (e.g. B-CLL)
CD7	Tp41 antigen/Fc receptor for IgM (FcμR)	gp41	Leu-9(γ2a)[b], OKT16(γ2)[c], 3A1(γ1)[d], DK24(γ2b)[f], PAN-L7D(γ2a)[g], HD49(γ1)[h], CLB-3A1(γ2a)[k], WT1(γ2a)[q]	almost all T cells, NK cells, subpopulation of immature myeloid cells (e.g. some AML)
CD8	T8 antigen; the CD8 molecule consists of two disulfide linked chains: α-α homodimer or α-β heterodimer/involved in MHC-class-I-restricted antigen recognition	gp32	Leu-2a(γ1)[b], OKT8(γ2a)[c,d], T8A(γ1)[e], CLB-T8/1(γ1)[k], DK25(γ1)[f], MID4(μ)[g], T811(γ)[g,p] BW135/80(γ2a)[h], Tü102(γ1)[o]	subpopulation of cortical thymocytes, cytotoxic/suppressor T cells, subpopulation of NK cells
		CD8-α	most CD8 antibodies	
		CD8-β	–	

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD9	p24 antigen/induction of aggregation of platelets	p24	BA-2(γ3)[r], CLB-thromb/8(γ2a)[k], FMC8(γ2a)[g]	subpopulation of precursor B cells, subpopulation of B cells (follicular center cells), monocytes, megakaryocytes, platelets, eosinophils, basophils
CD10	common ALL antigen (CALLA)/neutral endopeptidase (enkephalinase)	gp100	J5(γ2a)[e], VIL-A1(μ)[h,i], BA-3(γ2b)[r], OKBCalla(γ2a)[c], W8E7(γ2a)[b], SS-2/36(γ1)[f], CALLA-LC3M(γ1)[g], CLB-CALLA/1(γ2a)[k], UL96(μ)[o]	subpopulation of precursor B cells, subpopulation of B cells (follicular center cells), subpopulation of cortical thymocytes, granulocytes
CD11a	LFA-1 antigen (αL chain); associated with CD18 antigen/adhesion molecule; binds to CD54 antigen (ICAM-1)	gp180	MHM24(γ1)[f], CLB-LFA-1/2(γ2a)[k]	majority of lymphoid and myeloid cells; no membrane expression in LAD patients
CD11b	MAC-1 antigen (αM chain); associated with CD18 antigen/adhesion molecule; CR3 (C3bi receptor)	gp155	Leu-15/D12(γ2a)[b], Ki-M5(γ1)[h] Mo1(μ)[e], CLB-mon-gran/1(μ)[k], 2LPM19c(γ1)[f], OKM1(γ2b)[c,d]	monocytes, macrophages, granulocytes, NK cells; no membrane expression in LAD patients
CD11c	p150,95 antigen (αX chain); associated with CD18 antigen/adhesion molecule	gp150	Leu-M5/SHCL3(γ2b)[b], KB90(γ1)[f], FD11(γ1)[g]	monocytes, macrophages, granulocytes, subpopulations of lymphocytes (e.g. HCL-like cells in the spleen and NK cells); no membrane expression in LAD patients
CDw12	myeloid antigen	p90-120?	M67(γ1)[s]	monocytes, granulocytes, platelets
CD13	pan-myeloid antigen/ aminopeptidase N	gp150	My7(γ1)[e], CLB-mon-gran/2(γ2a)[k], MCS2(γ)[t]	almost all myeloid cells, dendritic cells in the skin
CD14	monocytic antigen; PI-linked protein	gp55	Leu-M3(γ2b)[b], 63D3(γ1)[d], My4(γ1)[e], Mo2(μ)[e], FMC17(γ2b)[g], UCHM1(γ2a)[g], VIM-13(μ)[i], CLB-Mon/1(γ2a)[k]	monocytic cells, macrophages, follicular dendritic reticulum cells; absent in patients with PNH
CD15	X hapten; FAL (fucosyl-N-acetyl-lactosamine)	variety of gp's	VIM-D5(μ)[i], Leu-M1(μ)[b], CLB-gran/2(μ)[k], C3D-1(μ)[f], FMC13(μ)[g], VIM-C6(μ)[h], Tü9(μ)[o], MCS1(γ2a)[t], 1G10(μ)[u]	cells of the granulocytic lineage, weak expression by monocytes, Reed Sternberg cells
CD16	FcγRIII (low affinity Fc receptor for IgG); PI-linked protein on granulocytes	gp50-65	Leu-11b(μ)[b], OKNK(μ)[c], VEP-13(μ)[h], CLB-FcR-gr/1(γ2a)[k], GRM1(γ2a)[o]	neutrophil granulocytes, macrophages, NK cells; absent on granulocytes in patients with PNH
CDw17	lactosylceramide	glycolipid	T5A7(μ)[u]	granulocytes, monocytes, platelets
CD18	integrin β2 chain/ associated with CD11 chains (α chains)	gp95	MHM23(γ1)[f], CLB-LFA-1/1(γ1)[k]	majority of lymphoid and myeloid cells (see CD11); absent or defect in LAD patients
CD19	pan-B cell antigen/function in B cell activation	gp90	Leu-12(γ1)[b], B4(γ1)[e], CLB-B4(γ1)[k], HD37(γ1)[g,f,h,p], F97-4A2(μ)[g], B-ly-3(μ)[o]	precursor B cells and B cells
CD20	B cell antigen/function in B cell activation	p35	Leu-16(γ1)[b], B1(γ2a)[e], L26(γ2a)[f], B-ly-1(γ1)[o], G28-2(γ3)[v]	subpopulation of precursor B cells, all B cells, follicular dendritic reticulum cells; L26 recognizes an intracellular epitope of the CD20 molecule

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD21	B cell antigen/CR2 (C3d receptor); EBV receptor	gp140	B2(μ)[e], OKB1(γ2a)[c], HB-5(γ2a)[b], RFB6(γ)[w], F97-6B3(γ1)[g], B-ly-4(γ1)[o]	subpopulations of B cells (e.g. follicular mantle cells), follicular dendritic reticulum cells
CD22	B cell antigen/function in B cell activation; related to NCAM (CD56)	gp135	Leu-14/SHCL-1(γ2b)[b], HD6(γ1)[g,h,p], 4KB128(γ1)[f], RFB4(γ)[g,w], To15(γ2b)[f], HD39(γ1)[g,h,p], CLB-B-ly/1(γ1)[k]	precursor B cells and mature B cells
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	Leu-20/EBVCS-5(γ1)[b], Tü1(γ1)[o], MHM6(γ1)[f]	FcϵRIIa is expressed by a subpopulation of B cells (e.g. follicular mantle cells) and B-CLL cells; FcϵRIIb is expressed by monocytes and eosinophils
CD24	B cell-granulocytic antigen; PI-linked protein on granulocytes	gp42	BA-1(μ)[x], CLB-gran-B-ly/1(γ1)[k], OKB2(γ1)[c], VIB-E3(μ)[i], VIB-C5(μ)[i]	subpopulation of (precursor) B cells, granulocytes; absent on granulocytes in patients with PNH
CD25	Tac antigen/β chain of the IL-2 receptor (low affinity IL-2R)	gp55	2A3(γ1)[b], OKT26A(γ2)[c], IL-2R1(γ2a)[e], ACT-1(γ1)[f], CLB-IL-2R/1(γ2b)[k], Tü69(γ1)[o]	activated T cells, activated B cells (e.g. HCL), activated macrophages
CD26	dipeptidylpeptidase IV (DPP IV)	gp120	134-2C2(μ)[x]	activated T cells, activated B cells, macrophages
CD27	T cell antigen (homodimer)	p110 (55/55)	OKT18A(γ2)[c], VIT-14(γ2b)[i], CLB-9F4(γ2a)[k]	mature T cells, activated T cells, subpopulation of B cells (e.g. cytoplasmic expression in plasma cells)
CD28	Tp44 antigen(homodimer)/function in T cell proliferation	gp90 (44/44)	Kolt2(γ1)[t]	subpopulation of T cells (especially cytotoxic/suppressor T cells)
CD29	integrin β1 chain (VLA-β chain; platelet GPIIa)/associated with CDw49 molecules (VLA-α chains)	gp130	4B4(γ1)[e]	subpopulation of T cells (especially CD4 positive T cells), subpopulations of other leukocytes, megakaryocytes, platelets (broad tissue distribution)
CD30	activation antigen	gp105	Ki-1(γ3)[f], Ber-H2(γ1)[f]	subpopulation of activated lymphoid cells, Reed-Sternberg cells
CD31	myeloid antigen; platelet GPIIa′	gp140	SG134(γ1)[y]	monocytes, granulocytes, platelets, subpopulation of T cells
CDw32	FcγRII (intermediate affinity Fc receptor for IgG)	gp40	C1KM5(γ1)[z], IV.3(γ2b)[aa]	monocytes, subpopulation of macrophages, B cells, granulocytes, eosinophils
CD33	pan-myeloid antigen	gp67	Leu-M9(γ1)[b], My9(γ2b)[e],WM-54(γ1)[f], L4F3(μ)[u]	majority of myeloid and monocytic cells (except for granulocytes)
CD34	precursor antigen	gp115	My10(γ1)[b], BI-3C5(γ1)[g], ICH3(γ2a)[bb]	precursors of lymphoid cells, precursors of myeloid cells
CD35	CR1 (C3b receptor); four different allotypes	gp160-250	44D(γ1)[b], To5(γ1)[f], 7E11(γ1)[zz]	B cells, granulocytes, monocytes, erythrocytes, dendritic reticulum cells
CD36	monocytic-thrombocytic antigen/thrombospondin receptor (platelet GPIV)	gp90	OKM5(γ1)[c], ESIVC7(γ1)[k], 5F1(μ)[u], CIMeg1(γ1)[z]	monocytes, macrophages, early erythroid cells, megakaryocytes, platelets

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD37	B cell antigen	gp40-52	RFB7(μ)[g,w], Y29/55(γ2a)[cc], HD28(γ2a)[g,p], G28-1(γ1)[v]	B cells; weak expression on T cells, monocytes and granulocytes
CD38	T10 antigen	gp45	Leu-17(γ1)[b], OKT10(γ1)[c,d] A10(γ2a)[g]	activated T and B cells, precursor cells (e.g. thymocytes), subpopulations of B cells (e.g. follicular center cells), plasma cells
CD39	B cell antigen	gp80	OKT28(γ1)[c], G28-8(γ1)[v], G28-10(γ1)[v],	large part of mature B cells
CD40	B cell antigen/function in B cell activation	gp50	G28-5(γ1)[v]	subpopulation of B cells, interdigitating reticulum cells
CD41	platelet GPIIb-GPIIIa complex; platelet GPIIb consists of a large α subunit and a small β subunit (disulfide linked) and is associated with platelet GPIIIa (CD61 antigen)/ fibrinogen receptor	gp145/115	J15(γ2a)[f], CLB-thrombo/7(γ1)[k], HPL1(γ1)[g], V1-P11(γ1)[i]	megakaryocytes, platelets; absent or reduced in patients with Glanzmann's thrombasthenia
CD42a	platelet GPIX; associated with CD42b antigen (platelet GPIb)/receptor for von Willebrand factor	gp20	FMC25(γ1)[dd]	megakaryocytes, platelets; absent or reduced in patients with Bernard-Soulier syndrome
CD42b	platelet GPIb consists of a large α subunit and a small β subunit; associated with CD42a antigen (platelet GPIX)/receptor for von Willebrand factor	gp170 (143/22)	AN51(γ2a)[f], HPL11(γ1)[g],	megakaryocytes, platelets; absent or reduced in patients with Bernard-Soulier syndrome
CD43	leukosialin (sialophorin; leukocyte sialoglycoprotein)	gp95	Leu-22(γ1)[b], DFT-1(γ1)[f], MT1(γ1)[o], G10-2(γ1)[v], G19-1(γ1)[v]	T cells, monocytes, granulocytes (broad tissue distribution); absent in some patients with Wiskott-Aldrich syndrome
CD44	Pgp-1/lymphocyte homing receptor	gp80-95	Hermes-3(γ2a)[ee], BRIC35(γ1)[ff], NKI-P1(γ1)[gg]	leukocytes, e.g. T cells, granulocytes and erythrocytes (broad tissue distribution)
CD45	LCA (leukocyte common antigen), T200 antigen	gp180-220	anti-leukocyte (HLe-1)(γ1)[b], T200(γ1)[h] CLB-T200(γ1)[k], PD7/26(γ1)[f],	all leukocytes
CD45RO	restricted LCA	gp180	UCHL1(γ2a)[f]	thymocytes, activated T cells, memory T cells; weak expression on monocytes and granulocytes
CD45RA	restricted LCA	gp220	2H4(γ1)[e], 4KB5(γ1)[f], MT2(γ1)[o], MB1(γ1)[o], G1-15(γ1)[v]	virgin T cells, B cells, granulocytes, monocytes
CD45RB	restricted LCA	gp220 205,190	PD7/26(γ1)[f]	B cells, subpopulation of T cells, monocytes, macrophages, granulocytes
CD46	non-lineage antigen/ membrane cofactor protein (MCP)	gp66/56	J48(γ1)[hh]	leukocytes (broad tissue distribution)
CD47	non-lineage antigen	gp47-52	CIKM1(γ1)[z]	leukocytes (broad tissue distribution)

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD48	non-lineage antigen; PI-linked protein	gp41?	Tü145(μ)[o], J4-57(γ1)[hh]	leukocytes; absent in patients with PNH
CDw49b	VLA-α2 chain (platelet GPIa); associated with CD29/receptor for collagen	gp170	CLB-thrombo/4(γ1)[k]	platelets, activated T cells, cultured T cells
CDw49d	VLA-α4 chain; associated with CD29/fibronectin receptor; homing receptor for Peyer's patches	gp150	HP2/1(γ1)[ii], HP1/3(γ3)[ii]	thymocytes, T cells, B cells, monocytes
CDw49f	VLA-α6 chain (platelet GPIc); consists of a large α subunit and a small β subunit; associated with CD29/laminin receptor	gp150 (120/30)	GoH3(rat γ2a)[kk]	platelets, subpopulation of T cells
CDw50	non-lineage antigen	gp148/108?	–	leukocytes
CD51	αV chain of the vitronectin receptor (VNR-α); associated with CD61 (platelet GPIIIa)	gp140 (125/25)	NKI-M7(γ1)[ff]	platelets
CDw52	Campath-1 antigen	gp21-28?	YTH66.9(μ)[mm], YTH34.5(γ2b)[mm]	leukocytes (broad tissue distribution)
CD53	MEM-53 antigen	gp32-40	MEM-53(γ1)[nn], HD77(μ)[oo]	leukocytes
CD54	intracellular adhesion molecule-1 (ICAM-1)/ ligand for CD11a-CD18 molecule (LFA-1)	gp90	OKT27(γ2)[c], My13(γ1)[pp]	monocytes, lymphocytes (broad tissue distribution); increased expression upon activation
CD55	decay accelarating factor (DAF); PI-linked protein	gp70	BRIC110(γ1)[ff], BRIC128(μ)[ff]	leukocytes, platelets, erythrocytes (broad tissue distribution); absent in patients with PNH
CD56	neural cell adhesion molecule (NCAM); PI linked and transmembrane forms	gp135-220	Leu-19/NKH-1(γ1)[b]	NK cells, some T cells (neuroectodermal cells)
CD57	human natural killer cell antigen	gp110	Leu-7/HNK-1(μ)[b,d]	subpopulation of NK cells, subpopulation of T cells, some B cells
CD58	LFA-3; ligand for CD2 (LFA-2); PI-linked and transmembrane forms	gp40-65	G26(μ)[h], BRIC5(γ2a)[ff]	leukocytes, erythrocytes (broad tissue distribution)
CD59	Ly-6 antigen; PI-linked protein	gp18-20	MEM-43(γ2a),[nn] YTH53.1(γ2b)[mm]	leukocytes, platelets, erythrocytes (broad tissue distribution); absent in patients with PNH
CDw60	NeuAc-NeuAc-Gal; expressed by various gangliosides	glycolipid	M-T121(μ)[s], M-T32(μ)[s]	subpopulation of T cells, platelets
CD61	integrin β3 chain (platelet GPIIIa; VNR-β chain)/associated with platelet GPIIb (see CD41) or CD51(VNR-α)	gp115	Y2/51(γ1)[f], VIP-L2(γ1)[i] CLB-thrombo/1 (C17)(γ1)[k]	megakaryocytes, platelets

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
CD62	granule membrane protein (GMP-140); platelet GPIIa''	gp140	CLB-thrombo/5 (C8)(γ1),[k] CLB-thrombo/6 (C2) (γ1)[k]	megakaryocytes, platelets; increased expression upon activation
CD63	GP-53 antigen	gp53	CLB-gran/12 (γ1)[k]	activated platelets, monocytes, lymphocytes (weak), granulocytes (weak)
CD64	FcγRI (high affinity Fc receptor for IgG)	gp75	32.2 (γ1)[aa]	monocytes
CDw65	myelomonocytic antigen (fucoganglioside; ceramide-dodecasaccharide 40)	glycolipid	VIM-2(μ)[h,i], VIM-8(μ)[i]	majority of myeloid and monocytic cells and a part of their precursors
CD66	granulocytic antigen (phosphoprotein)	gp180-200	CLB-gran/10(γ1)[k]	granulocytes; increased expression upon activation
CD67	granulocytic antigen; PI-linked protein	p100	B13.9(γ1)[k]	granulocytes; increased expression upon activation; absent in patients with PNH
CD68	macrophage antigen	gp110	EBM11(γ1)[f], KP1 (γ1)[f], Ki-M6(γ1)[h], Ki-M7(γ1)[h]	macrophages (mainly cytoplasmic expression)
CD69	activation inducer molecule (AIM); early activation antigen	gp60 (28/34)	Leu-23/L78(γ1)[b]	early activated B and T cells, which do not yet proliferate
CDw70	activation antigen	?	Ki-24[qq]	subpopulation of activated B and and T cells, Reed-Sternberg cells
CD71	T9 antigen(homodimer)/ transferrin receptor	gp190 (95/95)	OKT9(γ1)[c], T9(γ2a)[e], Ber-T9(γ1)[f], VIP-1(γ1)[h,i]	proliferating cells (e.g. thymocytes), activated cells, macrophages
CD72	B cell antigen	gp43/39	J3-109(γ1)[hh], BU-40(γ2a)[rr]	precursor B cells and mature B cells
CD73	ecto-5'-nucleotidase (5'-NT); PI-linked protein	gp69	AD-2 (γ1)[ss], 7G2.2.11(γ2a)[tt]	most B cells, subpopulation of T cells; absent in some types of SCID
CD74	MHC-class-II-associated invariant chain	gp41/35/33	BU-43(μ)[rr], BU-45(γ1)[rr], LN2(γ1)[0]	subpopulation of precursor B cells, B cells, subpopulation of monocytes
CDw75	B cell antigen	p53?	OKB4(μ)[c], LN1(μ)[o]	subpopulation of mature B cells (mainly germinal center B cells), subpopulation of T cells (weak)
CD76	B cell antigen	p85/67?	HD66(μ)[oo], CRIS-4(μ)[x]	subpopulation of mature B cells (mainly mantle zone B cells), subpopulation of T cells
CD77	B cell antigen (globo-triaosylceramide; Gb3)	glycolipid	424/4A11(μ)[uu]	germinal center B cells, activated B cells; Burkitt lymphoma cells, CC and CB-CC lymphoma cells
CDw78	pan-B cell antigen	gp67?	Leu-21(μ)[b]	subpopulation of precursor B cells, resting B cells, subpopulation of macrophages

CD no.[a]	Name(s)/function	mol. mass	McAb (mouse isotype)	Reactivity with hematopoietic cells
–	TdT/function in Ig and TcR gene rearrangement (insertion of nucleotides at junction sites)	p58	conventional antisera[vv] and McAb (HTdT-1(γ1)[vv], HTdT-3(γ2a)[vv], HTdT-4(γ2a)[vv])	immature lymphoid cells, small part of precursors of myeloid cells, virtually all ALL and some AML
–	B cell antigen	gp105	FMC7(μ)[g,dd]	mature B cells
–	HCL antigen	gp144	B-ly-7(γ1)[ww]	small subpopulation of B cells ("HCL-specific")
–	SmIg (surface membrane immunoglobulin); IgM, IgD, IgG, IgA, IgE	mol. mass is dependent on Ig class	conventional antisera and McAb	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
–	CyIg (cytoplasmic immunoglobulin)	mol. mass is dependent on Ig class	conventional antisera and McAb	CyIg positive cells (immunoblasts, immunocytes and plasma cells)
–	weak Cyμ (weak cytoplasmic expression of μ chain)	gp70	selected anti-μ antisera	pre-B cells; only μ heavy chains are weakly expressed in the cytoplasm (no Ig light chains)
–	TcR-$\alpha\beta$ (classical TcR; TCR2)	gp80 (44/40)	WT31(γ1)[b] and BMA031(γ2b)[h] probably recognize non-polymorphic epitopes of TcR-$\alpha\beta$	TcR-$\alpha\beta$ is expressed by the majority of mature $CD3^+$ T cells
–	TcR-$\gamma\delta$ (alternative TcR; TCR1)	gp75 (44/42 or 55/42)	anti-TcR-γ/δ-1(γ1)[b] and TCRδ1(γ1)[xx] recognize non-polymorphic epitopes of TcR-$\gamma\delta$	TcR-$\gamma\delta$ is expressed by a minority of mature $CD3^+$ T cells
–	MPO (myeloperoxidase); MPO consists of two subunits	gp60/12	MPO-7(γ1)[f], CLB-MPO-1(γ2a)[k]	majority of cells of the myeloid lineage (granulocytic and monocytic cells)
–	monocytic antigen	gp75	Monocyte-2/61D3(γ1)[yy]	monocytic cells
–	macrophage antigen	gp25	RFD9(γ1)[w]	macrophages
–	GpA (glycophorin A)	gp41	VIE-G4(μ)[h,i], CLB-ery/1(γ1)[k]	erythroid cells
–	H antigen; backbone of ABO variable blood group proteins	variable	CLB-eryH/1(μ)[k]	erythroid cells
–	HLA-A, B, C, shared determinant/heavy chain of MHC-class I molecule	gp43	W6/32(γ2a)[d,f,g]	all nucleated cells
–	β2-microglobulin/light chain of MHC-class I molecule	gp12	L368(γ1)[b], FMC16(γ2a)[g]	all nucleated cells
–	HLA-DR, non-polymorphic antigen/MHC-class II molecule	gp29/34	L243(γ2a)[b,d], OKDr(γ2a)[c], DK22(γ2a)[f], RF-B-HLA-DR(μ)[g], LN3(γ2a)[o]	hematopoietic precursor cells, B cells, activated T cells, monocytic cells and macrophages
–	nuclear antigen in proliferating cells	?	Ki-67(γ1)[f]	proliferating cells during late G1, S, G2 and M phases of the cell cycle

Footnotes belonging to the Appendix (Summary of clustered and non-clustered immunologic markers)

a. CD = cluster of differentiation, as defined during the Leukocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989).
b. Becton Dickinson, San Jose, CA.
c. Ortho Diagnostic Systems, Raritan, NJ.
d. American Type Culture Collection, Rockville, MD.
e. Coulter Clone, Hialeah,FL.
f. DAKOPATTS, Glostrup, Denmark.
g. SeraLab, Crawley Down, UK.
h. Behring, Marburg, FRG.
i. Dr. W. Knapp and Dr. O. Majdić, Vienna, Austria.
k. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; in other countries: Janssen Biochimica, Beerse, Belgium.
l. Dr. P.C.L. Beverly, London, UK.
m. Dr. C. Terhorst, Boston, MA.
n. Dr. P. Anderson, Boston, MA.
o. Biotest, Dreieich, FRG.
p. Boehringer Mannheim, Mannheim, FRG.
q. Dr. W. Tax, Nijmegen, The Netherlands.
r. Hybritech, San Diego, CA.
s. Dr. P. Rieber, Munich, FRG.
t. Nichirei Co., Tokyo, Japan.
u. Dr. I. Bernstein, Washington, DC.
v. Dr. J.A. Ledbetter, Oncogen Corporation, Seattle, WA.
w. Royal Free Hospital, London, UK.
x. Dr. R. Vilella, Barcelona, Spain.
y. Dr. S.M. Goyert, Tel-Hashomer, Israel.
z. Dr. G.R. Pilkington, Melbourne, Australia.
aa. Medarex Inc., West Lebanon, NH.
bb. Dr. R.J. Levinsky, London, UK.
cc. Dr. H.K. Forster, Hoffman-La Roche, Basel, Switzerland.
dd. Dr. H. Zola, Bedford Park, Australia.
ee. Dr. E.C. Butcher, Stanford, CA.
ff. Dr. D. Anstee, Bristol, UK.
gg. Dr. C.G. Figdor, Amsterdam, The Netherlands.
hh. Dr. J.M. Pesando, Seattle, WA.
ii. Dr. F. Sanchez, Madrid, Spain.
kk. Dr. A. Sonnenberg, Amsterdam, The Netherlands.
ll. Dr. F. Hogervorst, Amsterdam, The Netherlands.
mm. Dr. H. Waldmann, Cambridge, UK.
nn. Dr. V. Horejsi, Prague, Czechoslovakia.
oo. Dr. G. Moldenhauser, Heidelberg, FRG.
pp. Dr. C.I. Civin, Baltimore, MD.
qq. Dr. H. Stein, Berlin, FRG.
rr. Dr. D.L. Hardie, Birmingham, UK.
ss. Dr. M. Cooper, Birmingham, AL.
tt. Dr. L. Thompson, La Jolla, CA.
uu. Dr. Brodin, Lund, Sweden.
vv. Supertechs, Bethesda, MD.
ww. Dr. S. Poppema, Edmonton, Canada.
xx. T Cell Sciences, Cambridge, MA.
yy. Bethesda Research Laboratories, Gaithersburg, MD.
zz. Dr. N.M. Hogg, London, UK.

— : Underlined McAb work on paraffin sections.

Abbreviations used in the Appendix:

AIM = activation inducer molecule
ALL = acute lymphoblastic leukemia
AML = acute myeloid leukemia
CALLA = common ALL antigen
CB-CC = centroblastic-centrocytic lymphoma
CC = centrocytic lymphoma
Cylg = cytoplasmic Ig
CLL = chronic lymphocytic leukemia
CR = complement receptor
DAF = decay accelerating factor
DPP IV = dipeptidylpeptidase IV
EBV = Epstein Barr virus
FAL = fucosyl-N-acetyllactosamine
FcγR = Fc receptor for IgG
FcϵR = Fc receptor for IgE
FcμR = Fc receptor for IgM
GMP = granule membrane protein
GP = glycoprotein
gp = glycoprotein
GpA = glycophorin A
HIV = human immunodeficiency virus
HCL = hairy cell leukemia
ICAM = intercellular adhesion molecule
Ig = immunoglobulin
IL-2 = interleukin 2
kDa = kilo Dalton
LAD = leukocyte adhesion deficiency
LCA = leukocyte common antigen
LFA = leukocyte function antigen
McAb = monoclonal antibody/antibodies
MCP = membrane cofactor protein
MHC = major histocompatibility complex
MPO = myeloperoxidase
mol. mass = molecular mass in kDa
NCAM = neural cell adhesion molecule
NK cell = natural killer cell
5′-NT = 5′-nucleotidase
p = protein
PI = phosphatidyl-inositol glycan
PNH = paroxysmal nocturnal hemoglobinuria
SCID = severe combined immunodeficiency
SmIg = surface membrane Ig
SRBC = sheep red blood cells
TcR = T cell receptor
TdT = terminal deoxynucleotidyl transferase
VLA = very late antigen
VNR = vitronectin receptor

Rotterdam, March 1990

J.J.M. van Dongen, H.J. Adriaansen, H. Hooijkaas
Department of Immunology, Erasmus University, Rotterdam, The Netherlands.

CHAPTER 2.3

IMMUNOLOGIC MARKER ANALYSIS OF CELLS IN THE VARIOUS HEMATOPOIETIC DIFFERENTIATION STAGES AND THEIR MALIGNANT COUNTERPARTS*

J.J.M. van Dongen, H.J. Adriaansen and H. Hooijkaas

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

The characterization of cells in the various hematopoietic differentiation stages can be performed morphologically (1-5). Additional characterization is obtained by immunologic marker analysis (6-12). The expression of a particular set of immunologic markers designates a cell to a particular differentiation stage. The various markers are detectable by means of antibodies or by rosette techniques (13-18).

Leukemias and non-Hodgkin lymphomas (NHL) can be regarded as malignant counterparts of cells in the various hematopoietic differentiation stages (6-12,19-24). Hematopoietic differentiation schemes as presented in Figure 1, are based upon knowledge concerning normal hematopoiesis as well as leukemias and NHL (1-12,19-24). Such differentiation schemes are hypothetical and cannot be complete. In addition, it should be realized that differentiation is a gradual procession of events and that differentiation stages are not as finite as suggested in the figure. However, such differentiation schemes provide an indication as to where the various leukemias and NHL can be located according to their maturation arrest (Figure 2). It is noteworthy that a maturation arrest is not always restricted to one differentiation stage, but that several differentiation stages can occur within one malignancy.

It will be discussed which cells express the various immunologic markers, how the expression of these markers can be detected and how immunologic markers can be used for the characterization of normal hematopoietic cells as well as leukemic and NHL cells.

HEMATOPOIETIC DIFFERENTIATION STAGES AND IMMUNOLOGIC MARKERS

Hematopoietic differentiation can be divided into a lymphoid and a myeloid lineage. The

* *Published in: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116. Updated for CD codes and several new immunologic markers.*

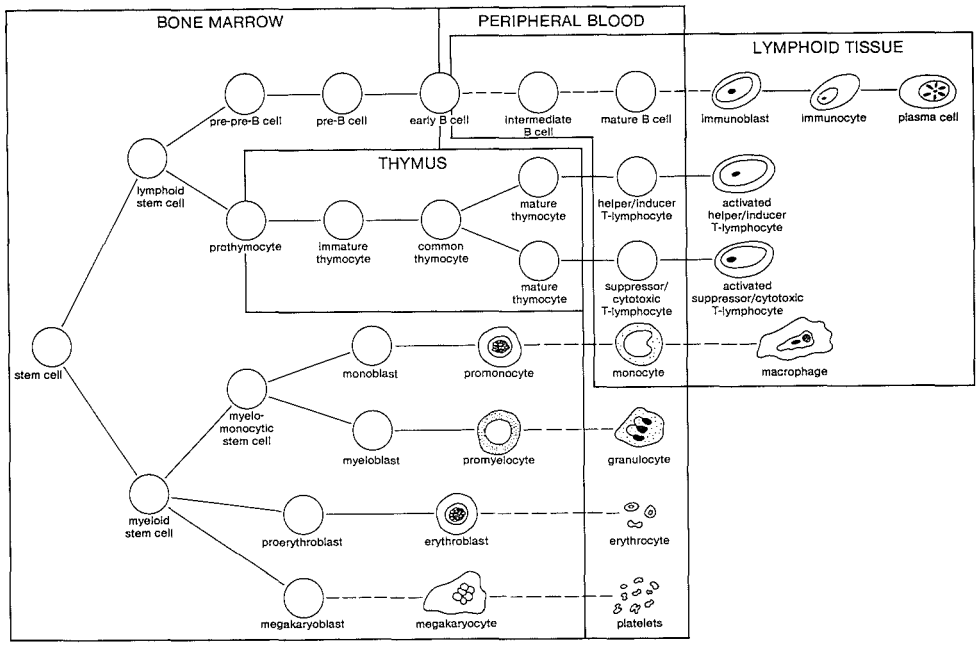
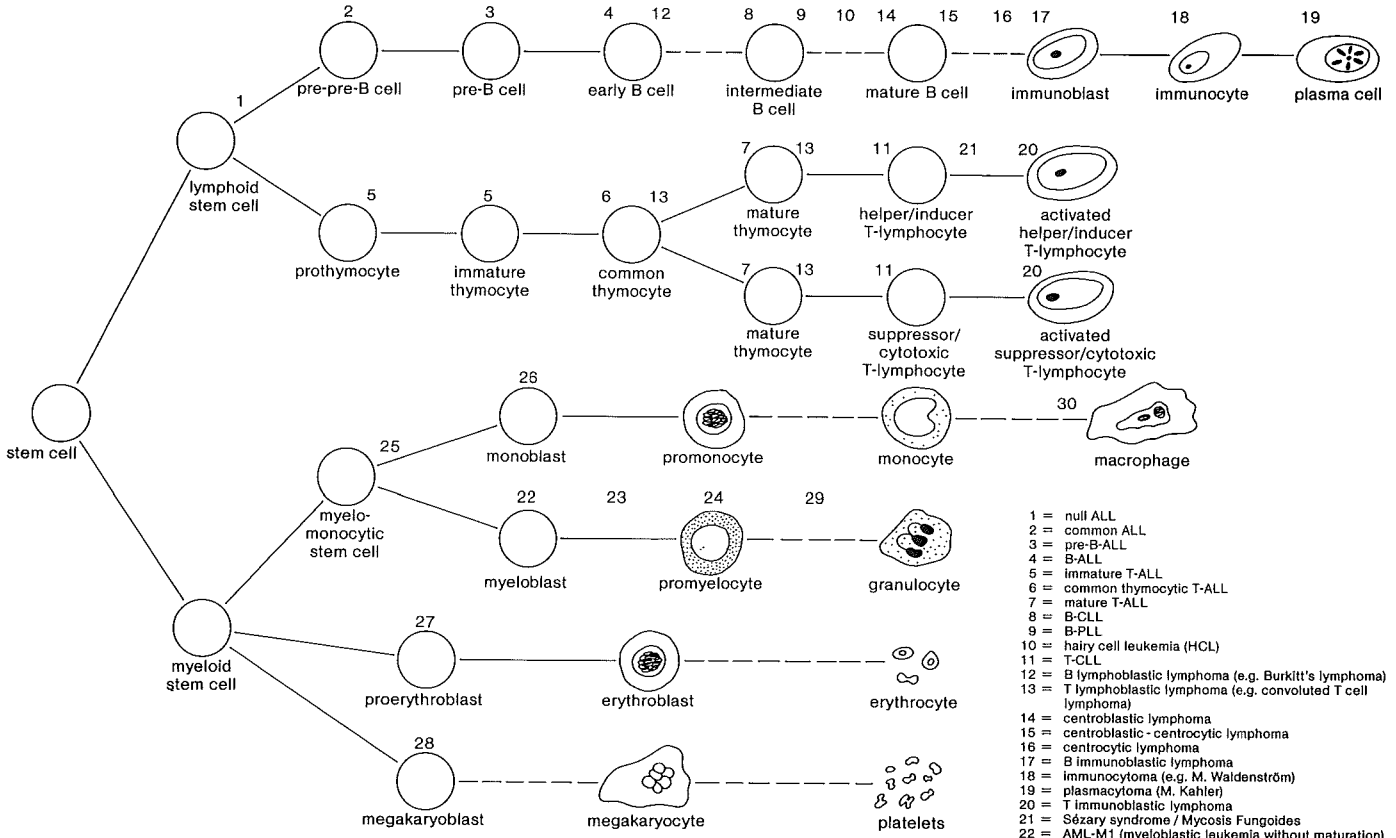


Figure 1. Hypothetical scheme of hematopoiesis. The different compartments, where cells in the various differentiation stages are located, are indicated.

lymphoid differentiation consists of the B cell and T cell lineages. The B cell differentiation occurs partly in the bone marrow (BM) and partly in the lymphoid tissues, finally resulting in antibody secreting plasma cells. The major part of the T cell differentiation probably occurs in the thymus, finally resulting in the mature helper/inducer T lymphocytes and suppressor/ cytotoxic T lymphocytes, which are present in the peripheral blood (PB) and lymphoid tissues. The precise origin of the natural killer (NK) cell is not yet known (not included in Figure 1). The myeloid differentiation, consisting of the monocytic, granulocytic, erythroid, and thrombocytic lineages mainly takes place in the BM (Figure 1).

Most hematopoietic differentiation stages can be characterized by the use of immunologic markers (6-12,19-24). Although immunologic markers represent differentiation antigens, they usually are not specific for one differentiation stage, but are expressed in several stages. However, a certain combination of markers can be specific for a particular differentiation stage. The expression of the various immunologic markers per differentiation stage is summarized in the Figures 3 and 4. As is illustrated by these figures, markers for the lymphoid differentiation lineage are more discriminating and more numerous than those for the myeloid lineage. Therefore, in particular, cells in the various lymphoid differentiation stages and their malignant counterparts can be characterized precisely. Detailed information about the markers presented in the Figures 3 and 4 is summarized in Table 1. In this table the markers are divided into groups according to their specificity: **1,** lymphoid markers (B cell markers, T cell markers) and NK cell markers; **2,** myeloid markers (pan-myeloid markers, myeloid-granulocytic markers,



1 = null ALL
2 = common ALL
3 = pre-B-ALL
4 = B-ALL
5 = immature T-ALL
6 = common thymocytic T-ALL
7 = mature T-ALL
8 = B-CLL
9 = B-PLL
10 = hairy cell leukemia (HCL)
11 = T-CLL
12 = B lymphoblastic lymphoma (e.g. Burkitt's lymphoma)
13 = T lymphoblastic lymphoma (e.g. convoluted T cell lymphoma)
14 = centroblastic lymphoma
15 = centroblastic - centrocytic lymphoma
16 = centrocytic lymphoma
17 = B immunoblastic lymphoma
18 = immunocytoma (e.g. M. Waldenström)
19 = plasmacytoma (M. Kahler)
20 = T immunoblastic lymphoma
21 = Sézary syndrome / Mycosis Fungoides
22 = AML-M1 (myeloblastic leukemia without maturation)
23 = AML-M2 (myeloblastic leukemia with maturation)
24 = AML-M3 (hypergranular promyelocytic leukemia)
25 = AML-M4 (myelomonocytic leukemia; AMML)
26 = AML-M5 (monocytic leukemia;AMol)
27 = AML-M6 (erythroleukemia)
28 = AML-M7 (megakaryoblastic leukemia)
29 = CML
30 = true histiocytic lymphoma

Figure 2. Hypothetical scheme of hematopoiesis. The numbers indicate as to where the various leukemias and NHL can be located according to their maturation arrest (see insert). The nomenclature of the NHL, indicated by numbers 12 to 20 is derived from the Kiel classification of NHL (4). The numbers 22 to 28 represent the various AML types according to the FAB classification (1-3).

monocyte-macrophage markers, erythroid markers and thrombocytic markers); **3,** markers which are expressed by cells of several differentiation lineages.

International nomenclature and classification of immunologic markers

In order to classify the large number of monoclonal antibodies (McAb) against immunologic markers, an international nomenclature has been developed analogous to nomenclatures such as those for classifying HLA antigens and blood group antigens (15-18). The various McAb (and the recognized immunologic markers) are classified in clusters of differentiation (CD). McAb against most lymphoid and myeloid markers have received a CD code (Table 1; Figures 3 and 4). Detailed information about the international CD nomenclature is summarized in Chapter 2.2 and an extensive list of all CD codes (CD1-CDw78) is given in the Appendix of Chapter 2.2. In the following sections the various McAb and the recognized markers will be indicated by their CD codes. When specific immunologic stainings are discussed, the names of the used McAb will be placed in parentheses behind the CD codes.

Lymphoid markers

The enzyme terminal deoxynucleotidyl transferase (TdT) is present in the nucleus of immature lymphoid cells, but is absent in more mature differentiation stages (6,7,25,26). Early in B cell differentiation, in the pre-B cell stage, weak cytoplasmic expression of μ immunoglobulin heavy chains (weak Cyμ) occurs (27-30), while more mature B cells express both immunoglobulin heavy and light chains on their cell surface membrane (SmIg) (31,32). Finally, the plasma cell strongly expresses cytoplasmic immunoglobulin (CyIg) heavy and light chains (33). Additional characterization of the various B cell differentiation stages can be performed by the use of other B cell markers such as the pan-B cell markers CD19 and CD22 (34-36), the common acute lymphoblastic leukemia antigen (CALLA; CD10 antigen) and the B cell markers CD9, CD20, CD21, CD24, CD37, FMC7 and B-ly-7 (39-50). The expression of the markers CD9 and CD24 is not restricted to the B cell lineage, but is also found in other differentiation lineages (17,18). The plasma cell is negative for the mentioned B cell markers, but expresses the CD38 antigen (Figure 3) (41).

During T cell differentiation, several T cell markers appear on the cell surface membrane, resulting in the expression of many T cell antigens by functional T lymphocytes (7,8,51,52). The putative prothymocyte only expresses the CD2 and CD7 antigens (53-58). The CD2 antigen represents the sheep red blood cell (SRBC) receptor (56,57,59). Another valuable marker for immature T cells is the cytoplasmic expression of the CD3 antigen (CyCD3) (60,61). During further differentiation also the CD5 antigen as well as the CD4 and CD8 molecules are expressed and finally the CD3 antigen appears on the cell surface membrane (51,52,62-66). The CD6 antigen is generally co-expressed with the CD5 antigen (67), while the CD1 antigen or so-called "common thymocyte antigen" is only temporarily expressed in the thymus (Figure 3) (51,52,68).

It is noteworthy that the mature T cell antigen CD3 is closely associated with the T cell

receptor (TcR) (69-71), which represents an antigen-specific receptor, comparable to SmIg, the antigen-specific receptor of the B cell (72-74). Within the protein complex formed by TcR and CD3(TcR-CD3 complex), the CD3 antigen probably plays an important role in the anchorage of TcR as well as in the transduction of signals from TcR to intracellular components (71,75). Two types of TcR exist: the "classical" TcR-$\alpha\beta$ and the "alternative" TcR-$\gamma\delta$ (76,77). TcR-$\alpha\beta$ is expressed on the majority of T lymphocytes, while TcR-$\gamma\delta$ is found on a minority of the $CD3^+$ T cells (78-81).

NK cells generally express the CD16 antigen (low affinity Fc receptor for IgG; FcγRIII) and the CD56 antigen (neural cell adhesion molecule; NCAM) (82). A subpopulation of the NK cells also expresses the CD8 molecule and the CD57 molecule (83). The CD16, CD56 and CD57 molecules may also be expressed by a subpopulation of $CD3^+$ T lymphocytes. The CD16 antigen is also expressed by granulocytes (84).

Myeloid markers

Analogous to the lymphoid markers, also during myeloid differentiation more or less characteristic markers appear on the cell surface membrane, while other markers disappear. Virtually all myelo-monocytic cells (immature and mature) are positive for the CD13 and CD33 molecules (9,11,85,86). Also CDw65 and myeloperoxidase (MPO) are expressed by most myelo-monocytic cells (87,88). Cells of the monocytic lineage express CD14 (85,86,89-91) and more mature monocytes are positive for Monocyte-2 (91,92), while macrophages may express the markers CD68 and RFD9 (93,94). The myeloid-granulocytic cells are positive for CD15 (95,96), while more mature granulocytic cells also express CD16, CD66 and CD67 (Figure 4) (17,18). The H antigen, which represents the backbone of the ABO blood group proteins, and glycophorin A (GpA) can be used as markers for cells of the erythroid lineage (Figure 4) (97-100). Cells of the megakaryocytic-platelet lineage can be recognized by use of McAb against the platelet-specific glycoproteins (GP), such as the whole GpIIb-GPIIIa complex (CD41 molecule), GPIX chain (CD42a molecule), GPIb (CD42b molecule) and GPIIIa chain (CD61 molecule) (Table 1 and Figure 4) (101-104).

Markers which are not or partly restricted to one differentiation lineage

Several markers are not restricted to one differentiation lineage, but are expressed by cells of several differentiation lineages. The HLA-DR antigen is expressed by cells in immature hematopoietic differentiation stages, but also by B cells, monocytic cells and activated T lymphocytes (Figures 3 and 4) (105-108). The interleukin 2 (IL-2) receptor (CD25 antigen) is expressed by activated T cells and activated B cells, while the CD38 antigen and the transferrin receptor (CD71 antigen) are expressed by virtually all proliferating and activated cells (17,18,108-110). The precursor antigen CD34 is found on most immature cells of both the lymphoid and myeloid differentiation lineages (Figures 3 and 4) (111).

The CD16 antigen (FcγRIII) is not only expressed on NK cells (82,83), but is also found on mature granulocytes (Figures 3 and 4) (84,112). The CD11/CD18 antigens represent a family

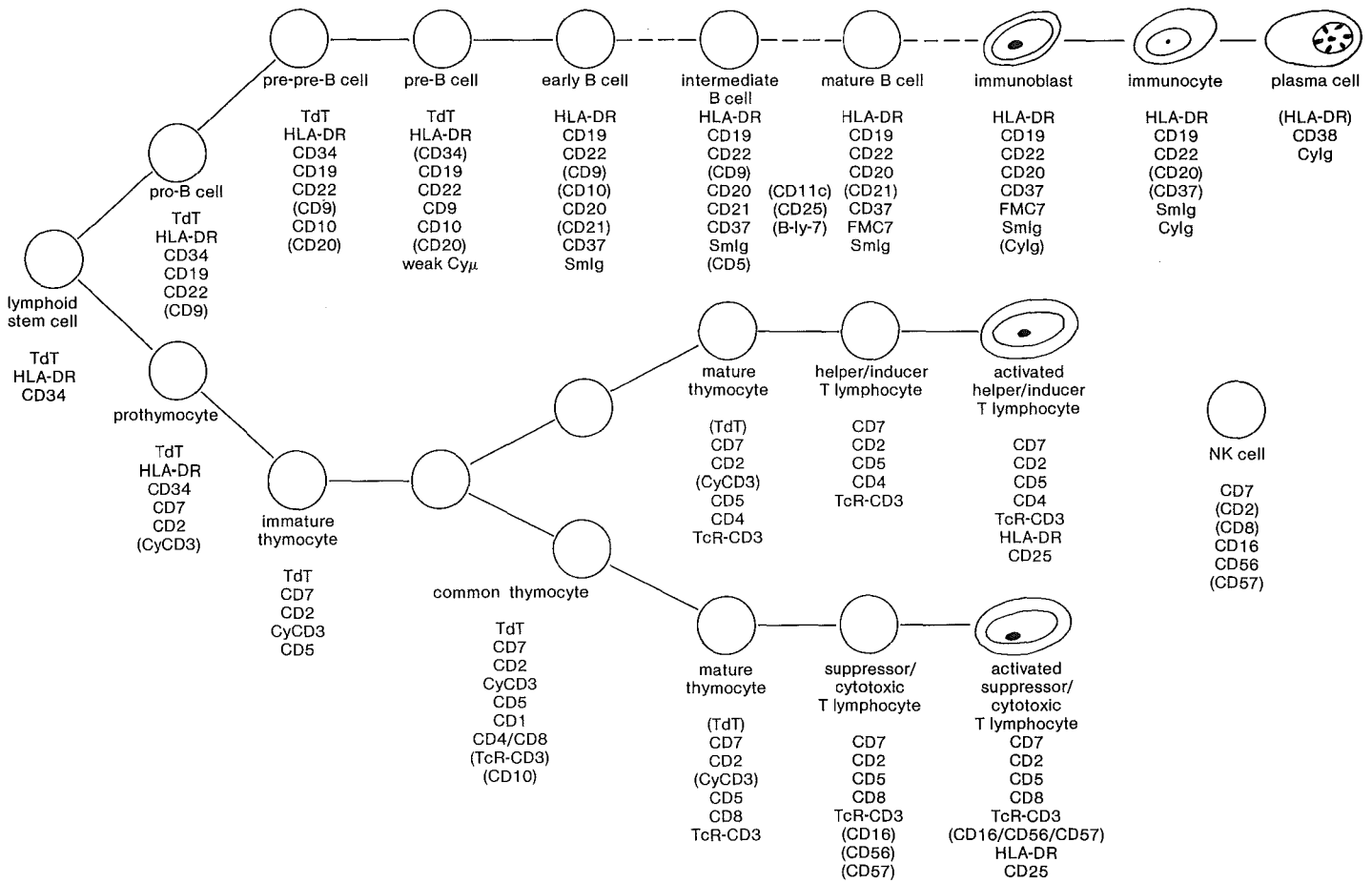


Figure 3. Hypothetical scheme of lymphoid differentiation. The expression of the various immunologic markers by cells in the different stages of lymphoid differentiation are indicated. The markers in parentheses are not always expressed.

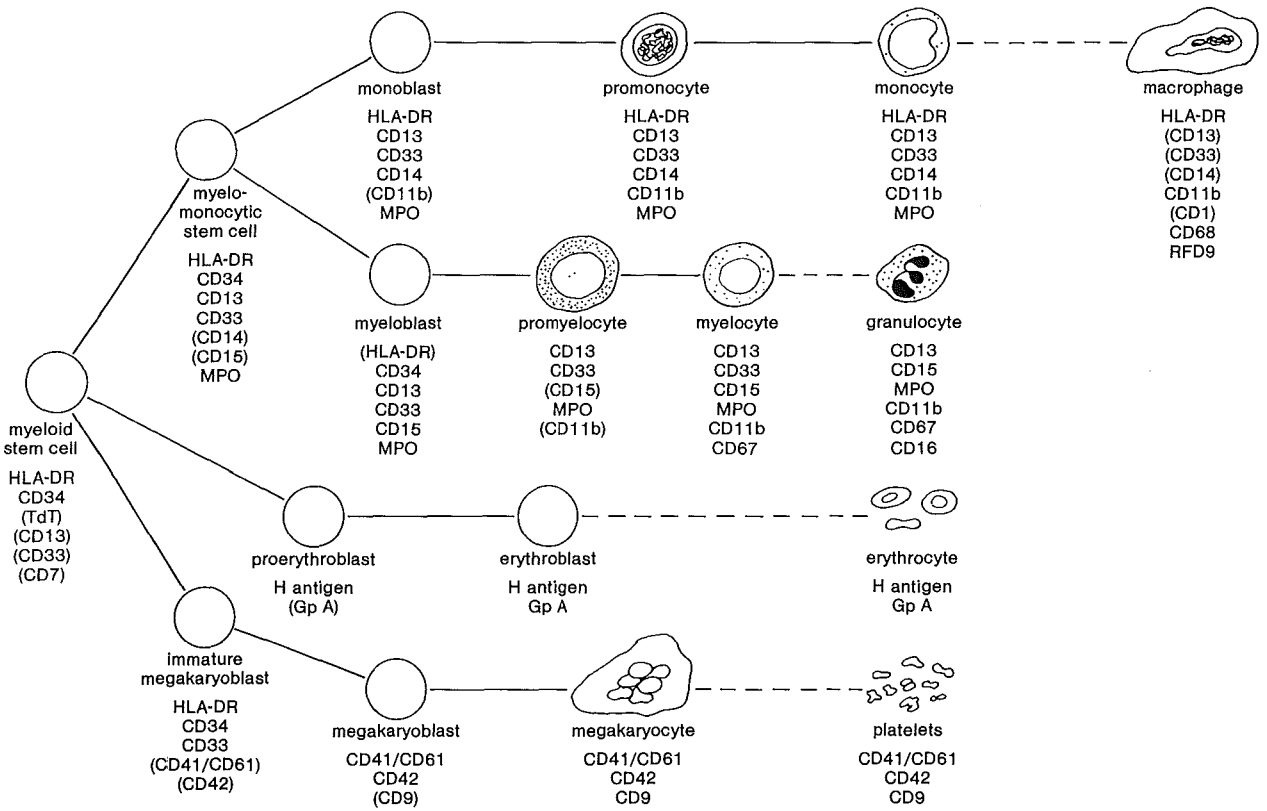


Figure 4. Hypothetical scheme of myeloid differentiation. The expression of the various immunologic markers by cells in the different stages of myeloid differentiation are indicated. The markers in parentheses are not always expressed.

TABLE 1. Immunologic markers, which can be used for the characterization of cells in the various hematopoietic differentiation stages and their malignant counterparts.

CD[a,b] code	Antibodies[b]/ techniques	Antigen recognized	Reactivity with hematopoietic cells	References
1. LYMPHOID MARKERS				
Immature lymphoid marker				
–	anti-TdT[c] antiserum	terminal deoxynucleotidyl transferase	immature lymphoid cells (Figure 3), a small subpopulation of immature myeloid cells (Figure 4)	6,7,25,26
B cell markers				
CD10	VIL-A1[d,e], J5[f], BA-3[g]	common ALL antigen (CALLA) (gp100)	pre-pre-B cells, pre-B cells, subpopulation of cortical thymocytes (Figure 3), granulocytes	6,37-41,122,123
CD19	B4[f], Leu-12[h]	pan-B cell antigen (gp90)	all (precursor) B cells (Figure 3)	34,40,41
CD20	B1[f], Leu-16[h]	B cell antigen (p35)	all B cells (Figure 3)	35,40,41,44,45
CD21	B2[f], HB-5[h]	B cell antigen; complement (C3d) receptor (CR2) (gp140)	subpopulations of B cells (Figure 3)	35,40,41,46
CD22	Leu-14[h], HD39[d,i,k], RFB4[i,o]	pan-B cell antigen (gp135)	all (precursor) B cells (Figure 3)	35,36,41
CD37	Y29/55[l]	B cell antigen (gp40-52)	$SmIg^+$ B cells (Figure 3)	35,41,47
–	FMC7[i,m]	B cell antigen (gp105)	subpopulations of $SmIg^+$ B cells (Figure 3)	40,41,48,49
–	B-ly-7[n]	hairy cell antigen (gp144)	small subpopulation of normal B cells; HCL	50
–	M rosette	mouse red blood cell (MRBC) receptor	subpopulation of B cells ("resting B cells"), B-CLL	118,121
–	weak Cyμ (pre-B)	weak cytoplasmic expression of μ heavy chain	pre-B cells (Figure 3)	27-30
–	SmIg ($\kappa,\lambda,\mu,\delta,\gamma,\alpha,\epsilon$)	surface membrane immunoglobulin	$SmIg^+$ B cells (each B cell expresses only one Ig light chain isotype: κ or λ, but can express multiple Ig heavy chain isotypes)	31,32
–	CyIg ($\kappa,\lambda,\mu,\delta,\gamma,\alpha,\epsilon$)	cytoplasmic immunoglobulin	immunoblasts, immunocytes, plasma cells	33
–	κ/λ ratio		the normal κ/λ ratio ranges from 0,8 to 2,0 (mean:1,4)	31
T cell markers				
CD1	Leu-6[h], 66IIC7[p], OKT6[q,r]	T6 antigen (common thymocyte antigen) (gp43, gp45, gp49)	cortical thymocytes (Figure 3), Langerhans cells, subpopulation of dendritic cells (Figure 4), subpopulation of B cells	51,52,68,115
–	E rosette	sheep red blood cell (SRBC) receptor	all T cells,most NK cells	56,57,59
CD2	T11[f], Leu-5b[h], OKT11[q,r]	T11 antigen; SRBC receptor (gp50)	all T cells,most NK cells (Figure 3)	51,52,56,57

CD[a,b] code	Antibodies[b]/ techniques	Antigen recognized	Reactivity with hematopoietic cells	References
CD3	VIT-3[e], Leu-4[h], OKT3[q,r], UCHT1[s,t]	T3 antigen (gp16-25)	immature T cells (cytoplasmic expression) and mature T cells (membrane expression (Figure 3)	51,52,60,61
CyCD3	Leu-4[h], UCHT1[s,t]	cytoplasmic CD3 antigen	cytoplasmic expression of the CD3 antigen in immature T cells (Figure 3)	60,61
CD4	T4A[f], Leu-3a[h], OKT4[q,r]	T4 antigen (gp60)	subpopulation of thymocytes, helper/inducer T cells (Figure 3)	51,52,64
CD5	T1B[f], Leu-1[h], OKT1[r]	T1 antigen (gp67)	thymocytes and mature T cells, subpopulation of B cells (Figure 3), B-CLL	51,52,62,63 117-119
CD6	OKT17[q]	T12 antigen (gp120)	thymocytes and mature T cells, subpopulation of B cells (B-CLL)	67,120
CD7	Leu-9[h], 3A1[r], WT1[u]	Tp41 antigen (gp41)	virtually all T cells, NK cells (Figure 3), subpopulation of immature myeloid cells (Figure 4), ~5% of AML	53-55
CD8	T8A[f], Leu-2a[h], OKT8[q,r]	T8 antigen (gp32)	subpopulation of cortical thymocytes, cytotoxic/suppressor T cells, subpopulation of NK cells (Figure 3)	51,52,64,65
–	BMA031[d], WT31[h]	TcR-$\alpha\beta$ (gp80)	majority (85-98%) of mature $CD3^+$ T cells	78,79
–	anti-TCR-γ/δ-1 (11F2)[h], TCRδ1[v]	TcR-$\gamma\delta$ (gp75)	minority (2-15%) of mature $CD3^+$ T cells	80,81
NK cell markers				
CD56	Leu-19(My31)[h]	neural cell adhesion molecule (NCAM) (gp135-220)	NK cells, some T cells (Figure 3)	82
CD57	Leu-7(HNK-1)[h,r]	human NK cell antigen (gp110)	subpopulation of NK cells, subpopulation of T cells (Figure 3)	83
2. MYELOID MARKERS				
Pan-myeloid markers				
CD13	My7[f], MCS2[w], CLB-mon-gran/2[x]	pan-myeloid antigen (gp150)	almost all myeloid cells (Figure 4)	9,11,85,86
CD33	My9[f], Leu-M9[h]	pan-myeloid antigen (gp67)	majority of myeloid and monocytic cells, except for granulocytes (Figure 4)	11
CDw65	VIM-2[d,e]	myelomonocytic antigen	majority of myeloid and monocytic cells	87
–	MPO-7[s], CLB-MPO-1[x]	myeloperoxidase (MPO) (gp60/12)	majority of myeloid and monocytic cells (Figure 4)	88
Monocyte-macrophage markers				
CD14	My4[f], Mo2[f], Leu-M3[h], FMC17[i], UCHM1[i]	monocytic antigen (gp55)	monocytic cells, macrophages (Figure 4)	9,11,85,86, 89-91

CD[a,b] code	Antibodies[b]/ techniques	Antigen recognized	Reactivity with hematopoietic cells	References
CD68	Ki-M6[d], Ki-M7[d]	macrophage antigen (gp110)	macrophages (Figure 4)	93
–	Monocyte-2/61D3[y]	monocytic antigen (gp75)	monocytic cells	91,92
–	RFD9[o]	macrophage antigen (gp25)	macrophages (Figure 4)	94
Myeloid-granulocytic markers				
CD15	VIM-D5[e], Leu-M1[h]	X hapten	cells of the granulocytic lineage (Figure 4)	95,96
CD66	CLB-gran/10[x]	granulocytic antigen (gp180-200)	granulocytes	18
CD67	B13.9[x]	granulocytic antigen (p100)	granulocytes (Figure 4)	18
Erythroid markers				
–	CLB-eryH[x]	H antigen (backbone of ABO proteins)	erythroid cells (Figure 4)	97
–	VIE-G4[d,e],CLB-ery/1[x]	GpA (glycophorin A) (gp41)	erythroid cells (Figure 4)	97-100
Megakaryocyte-platelet markers				
CD41	J15[s], CLB-thrombo/7[x]	GPIIb-GPIIIa complex (Glanzmann antigen) (gp145/115)	megakaryocytes, platelets (Figure 4)	101-104
CD42a	FMC25[m]	platelet GPIX (gp20)	megakaryocytes, platelets (Figure 4)	101-103
CD42b	AN51[s]	platelet GPIb (gp170)	megakaryocytes, platelets (Figure 4)	102,10
CD61	CLB-thrombo/1(C17)[x], Y2/51[s]	platelet GPIIIa (gp115); associated with GPIIb (see CD41)	megakaryocytes, platelets (Figure 4)	101-104
3. MARKERS WHICH ARE NOT RESTRICTED TO ONE DIFFERENTIATION LINEAGE				
CD9	BA-2[g], CLB-thrombo/8[x]	p24 antigens	subpopulation of precursor B cells, subpopulation of B cells (Figure 3), megakaryocytes, platelets (Figure 4)	39-43,101
CD11a	CLB-LFA-1/2[x]	LFA-1 antigen (gp180)	majority of lymphoid and myeloid cells	113,114
CD11b	Mo1[f], OKM1[q,r]	MAC-1 antigen, complement (C3bi) receptor (CR3)	monocytes, macrophages, granulocytes (Figure 4), NK cells	113,114
CD11c	Leu-M5/SHCL3[h]	p150,95 antigen	monocytes, macrophages, granulocytes, subpopulation of lymphocytes (Figure 3)	113,114
CD16	Leu-11b[h], CLB-FcR-gr[x]	low affinity Fc receptor for IgG (FcγRIII)	NK cells, subpopulation of T cells (Figure 3), neutrophil granulocytes (Figure 4)	82-84,112
CD18	CLB-LFA-1/1[x]	β chain of CD11 molecules	majority of lymphoid and myeloid cells	
CD24	BA-1[g]	B cell-granulocytic antigens (gp42)	subpopulations of (precursor) B cells, granulocytes	39-41,124

CD[a,b] code	Antibodies[b]/ techniques	Antigen recognized	Reactivity with hematopoietic cells	References
CD25	2A3[h]	β chain of interleukin 2 receptor (IL-2R)	activated T cells, activated B cells (e.g. HCL) (Figure 3)	17,18
CD34	My10[h], BI-3C5[i]	precursor antigen (gp115)	precursors of lymphoid and myeloid cells cells (Figures 3 and 4)	17,18,111
CD38	Leu-17[i], OKT10[q,r]	T10 antigen (gp45)	activated T and B cells precursor cells, subpopulations of B cells, plasma cells	17,18,41,108
CD71	T9[f], OKT9[q]	transferrin receptor (gp190)	proliferating cells, activated cells	108-110
–	L243[h,r], OKIa[q]	HLA-DR, non-polymorphic antigen (gp29/34)	hematopoietic precursor cells, B cells, activated T cells, monocytic cells (Figures 3 and 4)	105-108

a. CD = cluster of differentiation, as defined during the Leukocyte Typing Conferences (Paris, 1982; Boston, 1984; Oxford, 1986; Vienna, 1989).
b. Detailed information about all CD codes, additional clustered McAb and isotypes of McAb is given in the Appendix of Chapter 2.2. In this table we only included McAb, which are routinely used in the immunodiagnostic laboratory of the Department of Immunology, University Hospital Dijkzigt, Rotterdam, The Netherlands.
c. Supertechs, Bethesda, MD.
d. Behring, Marburg, FRG.
e. Dr. W. Knapp and Dr. O. Majdic, Vienna, Austria.
f. Coulter Clone, Hialeah,FL.
g. Hybritech, San Diego, CA.
h. Becton Dickinson, San Jose, CA.
i. SeraLab, Crawley Down, UK.
k. Boehringer Mannheim, Mannheim, FRG.
l. Dr. H.K. Forster, Hoffman-La Roche, Basel, Switzerland.
m. Dr. H. Zola, Bedford Park, Australia.
n. Dr. S. Poppema, Edmonton, Canada.
o. Royal Free Hospital, London, UK.
p. Monosan/Sanbio, Nistelrode, The Netherlands
q. Ortho Diagnostic Systems, Raritan, NJ.
r. American Type Culture Collection, Rockville, MD.
s. DAKOPATTS, Glostrup, Denmark.
t. Dr. P.C.L. Beverly, London, UK.
u. Dr. W. Tax, Nijmegen, The Netherlands.
v. T Cell Sciences, Cambridge, MA.
w. Nichirei Co., Tokyo, Japan.
x. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; in other countries: Janssen Biochimica, Beerse, Belgium.
y. Bethesda Research Laboratories, Gaithersburg, MD.

of adhesion molecules, in which the α chains (CD11a, CD11b and CD11c antigens) are associated with one common β chain (CD18 antigen) (113,114). CD11a/CD18 molecule represents leukocyte function antigen-1 (LFA-1), CD11b/CD18 represents the complement (C3bi) receptor type 3 (CR3), while the last member of this adhesion molecule family (CD11c/CD18) also binds complement (C3bi) (113,114). The expression patterns of the CD11/CD18 molecules are summarized in Table 1 and partly indicated in Figures 3 and 4 (113,114).

Some markers, which initially seemed to be restricted to one differentiation lineage, later appeared to be expressed by cells of other lineages as well. This is especially the case for several T cell markers. The CD1 antigen is not only expressed by thymocytes, but also by Langerhans cells in the skin (115). The CD4 molecule is also found on a subpopulation of monocytes and macrophages (116). The CD5 and CD6 antigens appear to be expressed by a subpopulation of B cells (117-120), which also express the mouse red blood cell (MRBC) receptor (118,121). Finally, the CD7 antigen is expressed by a small subpopulation of immature myeloid cells (54). Also some B cell markers are not totally lineage-restricted. For example, the CD9 antigen is also expressed by cells of the megakaryocytic-platelet lineage (101), CD10 molecules are found to be present on many cortical thymocytes (122) and on granulocytes (123) and also the CD24 antigen is found on granulocytes (124).

DETECTION OF IMMUNOLOGIC MARKERS OF CELLS IN SUSPENSION AND CELLS IN TISSUE SECTIONS

In recent years McAb have increasingly been used for the detection of immunologic markers in addition to conventional antisera (14-18). In order to visualize the reactivity of the antibodies, fluorochromes or enzymes are generally used as labels (125,126). Sometimes rosette techniques using erythrocytes are applied, e.g. for the detection of the T cell specific SRBC receptor (E rosette) (59) and the MRBC receptor (M rosette), which is expressed by a subpopulation of B cells (118,121). The staining techniques mentioned can be used for the detection of immunologic markers on hematopoietic cells in suspension (Figures 5-12) as well as on cells in tissue sections.

Immunologic marker analysis of cells in suspension

Cells in suspension can be obtained from BM, PB, cerebrospinal fluid (CSF), bronchoalveolar lavage (BAL) fluid, pleural exudate, ascites, synovial fluid, etc. Generally, the granulocytes and erythrocytes are removed from the BM and PB samples by ficoll density centrifugation, which purification facilitates the analysis of the remaining mononuclear cells (MNC) (127).

For the detection of markers on the cell surface membrane the cells are incubated with the relevant antibodies in suspension, while for the detection of cytoplasmic or nuclear markers, cytocentrifuge preparations are made, which are subsequently incubated with the relevant anti-

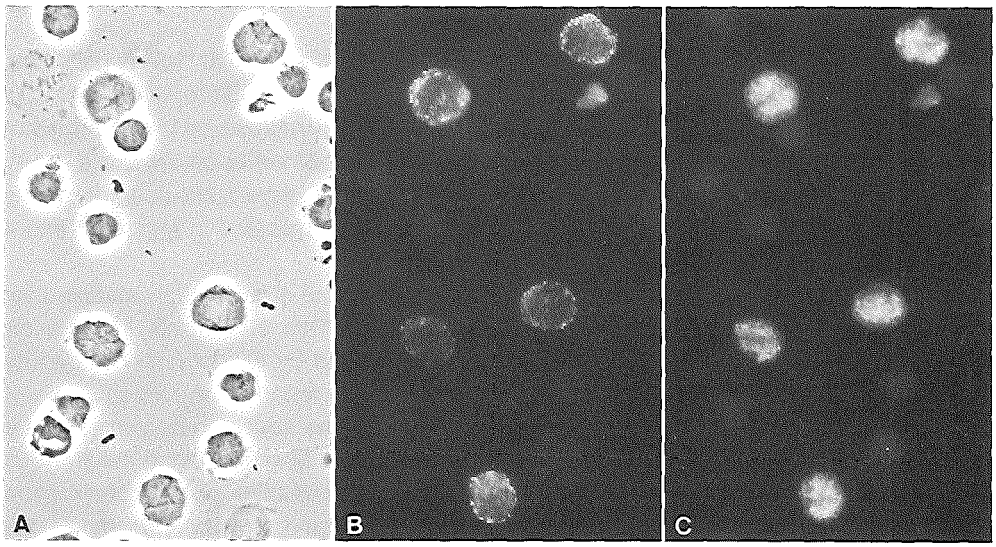


Figure 5. Double immunologic staining for CD10 and TdT on MNC of PB from a patient in early relapse of an ALL. **A:** phase contrast picture; **B:** CD10 positive cells (TRITC labeled); **C:** TdT positive cells (FITC labeled). All TdT positive cells in the PB sample appeared to express CD10, indicating that it concerned a CD10 positive ALL.

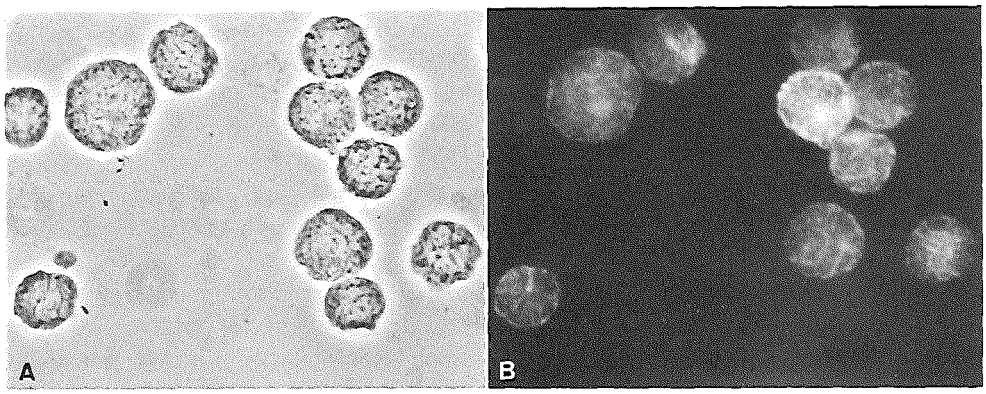


Figure 6. Immunologic staining for cytoplasmic μ Ig heavy chain on MNC of BM from an ALL patient at diagnosis. **A:** phase contrast picture; **B:** weak Cyμ positive cells (FITC labeled). Most MNC of BM appeared to be weakly positive for Cyμ, indicating that it concerned a pre-B-ALL.

bodies. In our laboratory we mainly use direct and indirect fluorescence methods for the visualization of the reactivity of the antibodies (Figures 5-7 and 9-12). It is one of the advantages of a fluorochrome as label, that double stainings can be easily performed and evaluated (Figures 5,7,9 and 10) (6-8,58). In these double stainings we use fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) as labels. TRITC can be replaced by texas red (TX) or phycoerythrin (128,129). Although phycoerythrin has advantages for fluorescence activated cell sorting procedures, we prefer to use TRITC in fluorescence microscopy, because phycoerythrin fades very quickly. If necessary, even a triple immunologic staining can be performed using colloidal gold particles as the third label next to FITC and TRITC (Figure 12) (125). Such double and triple immunologic stainings allow the evaluation of two or three different markers at the single cell level. This is important for determining whether the markers are expressed by the same cell or by different cells (125,130). Detailed information concerning methods for immunologic marker analysis of cells in suspension is described below.

In our opinion it is necessary to use phase contrast facilities on a fluorescence microscope, since phase contrast morphology gives valuable information about the cells evaluated and allows the precise localization of the labels, which is important when intracellular antigens are stained such as TdT (Figures 5,9 and 12). In addition, this equipment allows optimal discrimination between labeled cells and contaminating auto-fluorescent particles (for details, see Chapter 2.1).

Immunologic marker analysis of cells in tissue sections

Cells in tissues such as obtained by biopsies from lymph nodes, tonsils, liver and spleen, can be characterized immunologically by the use of tissue sections. The tissue sections, usually frozen sections, are incubated with the relevant antibodies. The reactivity of the antibodies is

visualized by the use of direct or indirect staining methods using an enzyme as label, such as peroxidase (PO) or alkaline phosphatase (AP) (20,131). Such enzymes transform a substrate into a colored precipitate. Double immunologic stainings, using enzymes as labels (e.g. PO and AP) are possible if the detected antigens are expressed by different cells (126,130-132). Expression of two antigens by the same cell is difficult to prove by use of enzymes as labels (130), unless the antigens are expressed very strongly (133,134). Detailed information concerning methods for immunologic marker analysis of cells in tissue sections are extensively described in several reviews (135-137).

The advantage of the use of tissue sections is that histologic information can be combined with information about the expression of immunologic markers (20). Nevertheless it may be necessary that a cell suspension is made from a tissue biopsy in order to perform immunologic marker analysis, especially if a part of the cell suspension is used for cytogenetic analysis.

METHODS FOR IMMUNOLOGIC MARKER ANALYSIS OF CELLS IN SUSPENSION

Preparation of cell suspensions

MNC from BM and PB are isolated by ficoll density centrifugation (Ficoll Paque; density 1.077 g/ml; Pharmacia, Uppsala, Sweden) for 15 min at room temperature (RT) with a centrifugal force of 1000 g. All standard washings of cells in suspension derived from BM, PB, CSF, pleural exudate, ascites, etc., are performed with phosphate-buffered saline (PBS) (300 mosmol; pH 7.8), supplemented with 0.5% bovine serum albumin (BSA; Organon Teknika, Oss,

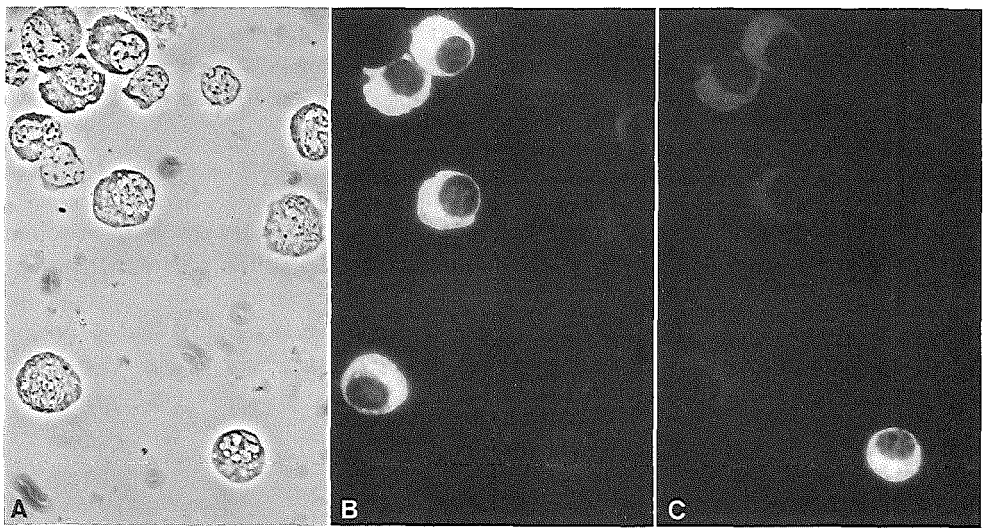


Figure 7. Double immunologic staining for Cyκ and Cyλ on BM cells from a patient with a plasmacytoma. **A:** phase contrast picture; **B:** Cyκ positive cells (TRITC labeled); **C:** Cyλ positive cell (FITC labeled). The majority of the Cylg positive cells appeared to be positive for Cyκ; only a few Cyλ positive cells were detectable in the cytocentrifuge preparation. This indicates that the plasmacytoma cells were Cyκ positive.

The Netherlands) and 0.05% sodium azide. Washing centrifugations are performed for 5 min at 4°C with a force of 400 g. If the cells are incubated in suspension for the detection of surface membrane markers by use of antibodies, the cell concentration is adjusted to 10^7 cells per ml. Cytocentrifuge preparations are made using cell suspensions with a concentration of 0.5 to 2 x 10^6 cells per ml, depending on the size of the cells; in the case of a BM or PB cell sample a concentration of 2 x 10^6 cells per ml is generally used. If the cell suspension is used for rosette techniques, the cell concentration is also adjusted to 2 x 10^6 cells per ml.

General remarks concerning immunologic stainings

Most immunologic markers are detected by the use of antibodies. Commercially available McAb generally can be diluted 1:50 to 1:200, while commercially available conventional antisera can be diluted 1:20 to 1:80. However, each batch of antibodies has to be optimally titrated for the different applications. The labeled cells or cytocentrifuge preparations are mounted in glycerol/PBS (9:1), containing 1 mg *p*-phenylenediamine per ml (BDH Chemicals, Poole, UK) to prevent fading of fluorochromes (138). Coverslips are sealed with paraffin wax with ceresin (BDH Chemicals). Some immunologic markers are detected by rosette techniques. Such rosette tests generally are evaluated by the use of a counting chamber.

We will describe several single, double and triple immunofluorescence staining methods as well as the microscopes, which we use for the evaluation of the immunofluorescence stainings.

Single immunologic stainings

In single staining methods using antibodies, we generally use FITC as fluorochrome. Several single staining methods using antibodies or rosette techniques are described below.

Detection of cell surface membrane markers by the use of antibodies.

In case of a direct immunologic staining (e.g. using FITC-conjugated anti-human-Ig antisera) 50 µl of a cell suspension (10^7 cells per ml) is incubated for 30 min at 4°C with 50 µl of the relevant, optimally titrated, FITC-conjugated antibody. After this incubation the cells are washed twice. In the case of an indirect immunologic staining (e.g. using mouse McAb), the cells undergo an additional incubation (30 min, 4°C) with the relevant FITC-conjugated second step antiserum (a FITC-conjugated goat anti-mouse-Ig antiserum, if mouse McAb are used). After the second incubation, the cells are washed twice and the cell pellet is mounted in glycerol.

Detection of CyIg.

Fifty µl of a cell suspension (2 x 10^6 cells per ml) is centrifuged on slides in a cytocentrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands). The cytocentrifuge preparations are air-dried for at least 15 min and the location of the cells is marked by encircling with a glass pencil. The preparations are fixed in acid ethanol (ethanol with 5% acetic acid) for 15 min at -20°C. The preparations are then washed in PBS (pH 7.8) for 15 min, dried,

and incubated with 15 µl of a FITC-conjugated anti-human-Ig heavy or light chain antiserum (Table 1) in a moist chamber (30 min, room temperature (RT)) (33). Subsequently the slides are washed again in PBS (15 min), mounted in glycerol, covered with a coverslip and sealed (Figures 6, 7 and 10).

Detection of TdT.

Cytocentrifuge preparations are made, air dried, fixed in methanol (30 min, 4°C) and washed in PBS. The preparations are incubated with 15 µl of optimally titrated rabbit anti-TdT antiserum (Table 1) in a moist chamber (30 min, RT) and washed in PBS. Subsequently, the preparations are incubated with 15 µl of a FITC-conjugated goat anti-rabbit-Ig antiserum and washed again (26,58). Afterwards the preparations are mounted in glycerol, covered with a coverslip and sealed (Figures 5, 9 and 12).

E rosette test.

SRBC are washed five times with PBS; washing centrifugations are performed for 10 min at RT with a centrifugal force of 1500 g. Subsequently, the SRBC are incubated with 2-amino-ethylisothiouronium bromide (AET) (40 mg per ml; pH 9.0) for 15 min at 37°C. Afterwards these AET-treated SRBC are washed several times with PBS and finally are resuspended in PBS, supplemented with 10% fetal calf serum (FCS) (59).

One hundred µl of the cell suspension (2×10^6 cells per ml) is mixed with 100 µl AET-treated SRBC (2×10^8 SRBC per ml), and centrifuged for 5 min at 4°C with a centrifugal force of 300 g. The pellet is incubated for at least 15 min at 4°C. After this incubation the pellet is very carefully resuspended in the supernatant. Fifty µl of acridine orange (5 µg per ml) is added to stain the DNA of the nucleated cells. The cell suspension is carefully mounted into a

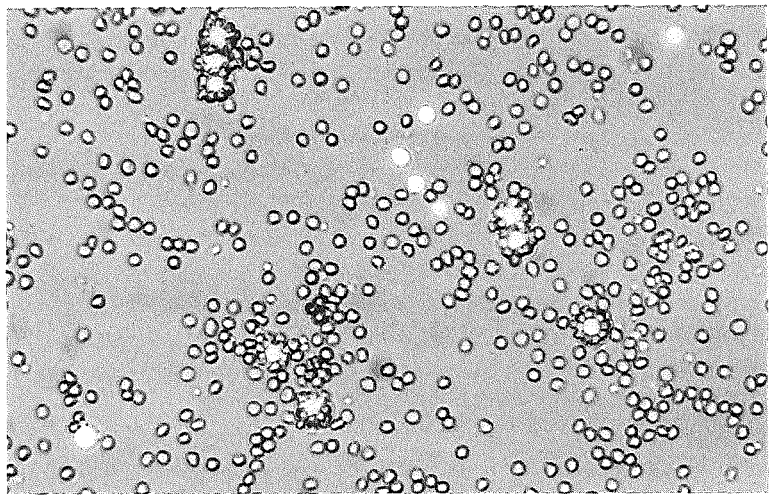


Figure 8. E rosette test on MNC of PB from a healthy adult. The nucleated cells were stained with acridine orange and evaluated by combining epi-illumination (using a FITC filter combination) with trans-illumination. Eight cells in the presented field form rosettes, while six cells do not.

counting chamber and evaluated by combining epi-illumination (using the FITC filter combination) with trans-illumination (Figure 8). A nucleated cell is regarded as positive, if three or more erythrocytes are attached to the cell (Figure 8).

M rosette test.

Freshly collected MRBC are washed five times with PBS. Fifty μl of the cell suspension (2 x 10^6 cells per ml), 50 μl of the MRBC suspension (1 x 10^8 MRBC per ml) and 100 μl FCS are mixed. This mixture is incubated for 15 min at 37°C and subsequently centrifuged for 5 min at 4°C with a centrifugal force of 300 g. The pellet is incubated at 4°C for 18 hours without being disturbed (118,121). After this incubation 100 μl of the supernatant is removed and the pellet is very carefully resuspended. Acridine orange is added to the cell suspension and the percentage M rosette forming cells is determined in a counting chamber (see E rosette test).

Double immunologic stainings

All of the various single immunologic stainings, described above, can be combined. When antibodies are used in double immunologic stainings, we use FITC and TRITC as labels. In our laboratory the double stainings with antibodies are performed in four different ways:

1. *Combination of two fluorochrome-conjugated antibodies.*
   The advantage of fluorochrome-conjugated antibodies is that these antibodies can be mixed and that therefore only one incubation step is needed for the double staining. However, weakly expressed antigens might be insufficiently stained in a direct staining method, so that positivity is not always detectable.

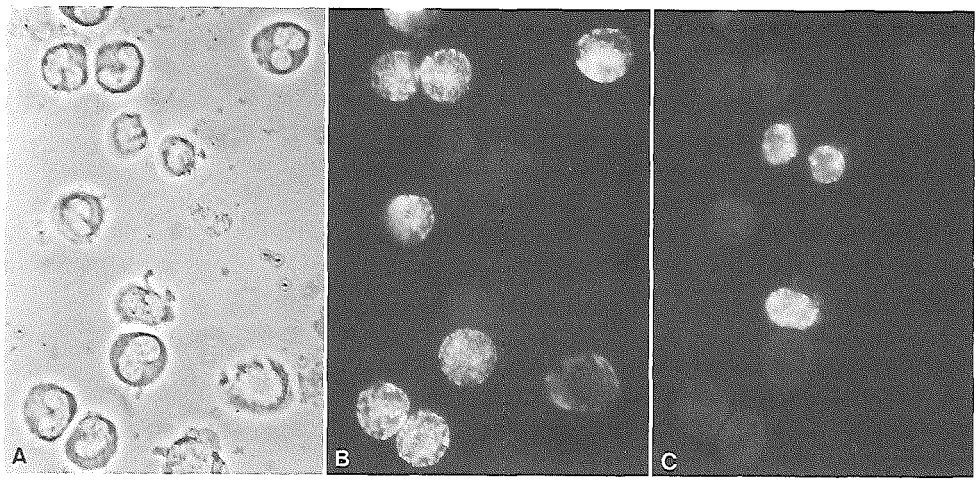


Figure 9. Double immunologic staining for the CD15 antigen and TdT on MNC of BM from a CML patient in early lymphoid BC. **A:** phase contrast picture; **B:** CD15 (VIM-D5) positive cells (TRITC labeled); **C:** TdT positive cells (FITC labeled). The three TdT positive cells do not express the myeloid marker CD15 (VIM-D5).

2. *Combination of a fluorochrome-conjugated mouse McAb and an unconjugated mouse McAb.*
   In such combinations, the cell suspension is first incubated with the unconjugated McAb, followed by an incubation with a fluorochrome-conjugated anti-mouse-Ig antiserum. Subsequently, the cells are incubated with normal mouse serum to block free antigen binding sites of the anti-mouse-Ig antiserum. Finally, the cells are incubated with the fluorochrome-conjugated McAb.
3. *Combination of two unconjugated mouse McAb of different isotypes.*
   Indirect staining methods using McAb can be performed by selecting pairs of mouse McAb on the basis of their different isotypes (e.g. IgM and IgG or IgG1 and IgG2). The isotypes of many clustered and non-clustered McAb are given in the Appendix of Chapter 2.2. The application of this double staining method is restricted by the availability of FITC- and TRITC-conjugated anti-mouse-Ig-(sub)class specific antisera (excellent antisera can be purchased from Nordic Immunological Laboratories).
4. *Combination of antibodies from different species.*
   Antibodies, raised in different species, can be combined very easily. E.g. a rabbit antiserum can be combined with a mouse McAb, using a FITC-conjugated goat anti-rabbit-Ig antiserum and a TRITC-conjugated goat anti-mouse-Ig antiserum, respectively.

By the use of these four possibilities or their combinations, many different double immunofluorescence stainings can be performed. Additional possibilities for double immunologic stainings can be obtained by using biotin-conjugated or arsanilic-acid-conjugated McAb; the reactivity of such McAb is visualized by the use of fluorochrome-conjugated avidin molecules or fluorochrome-conjugated anti-arsanilic-acid antisera, respectively (139,140). In the combined detection of a cell surface membrane antigen and a cytoplasmic antigen, we prefer to use FITC as label for the detection of the cytoplasmic antigen and TRITC as label for the detection of the cell surface membrane antigen. In our experience the use of TRITC for the labeling of cytoplasmic antigens generally results in a higher background staining as compared with FITC. In addition to double stainings with antibodies, an antibody staining can be combined with a rosette technique. Several double immunologic stainings will be described.

Double immunofluorescence staining for Smκ and Smλ.

The cell suspension is incubated with a mixture of a TRITC-conjugated anti-human-κ antiserum and a FITC-conjugated anti-human-λ antiserum (Table 1) (30 min at 4°C). After the incubation the cells are washed and a suspension preparation is made.

Double immunofluorescence staining for Cyκ and Cyλ.

A cytocentrifuge preparation is made, fixed in acid ethanol and subsequently incubated with 15 μl of a mixture of a TRITC-conjugated anti-human-κ antiserum and a FITC-conjugated anti-human-λ antiserum (30 min, RT) (Figure 7).

Double immunofluorescence staining for a cell surface membrane antigen and TdT.

Fifty μl of the cell suspension is incubated with the relevant antibody, which is directly or indirectly labeled with TRITC. After the incubation and washings, 200 μl of PBS/0.5% BSA is

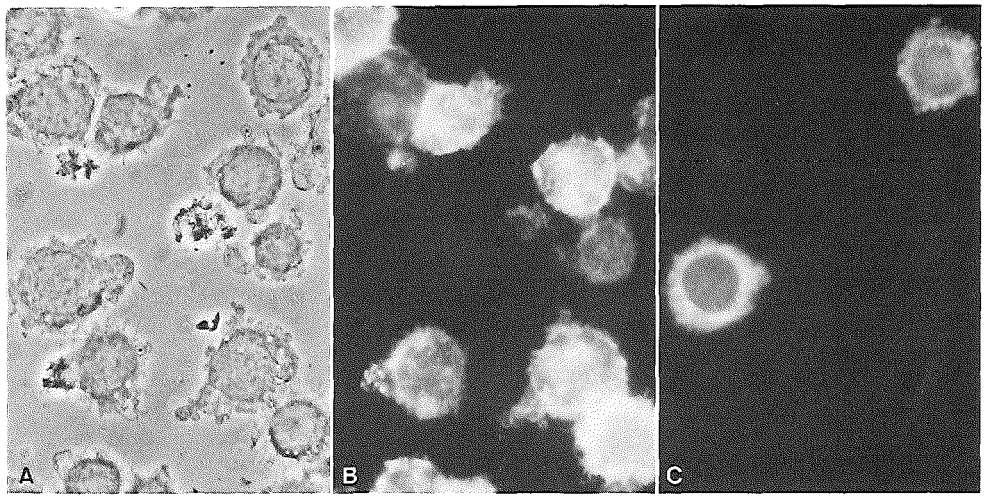


Figure 10. Double immunologic staining for HLA-DR and Cyμ on cells of a Burkitt lymphoma derived cell line. **A:** phase contrast picture; **B:** HLA-DR positive cells (TRITC labeled); **C:** Cyμ positive cells (FITC labeled). The HLA-DR positive cells did not express Cyμ.

added to the cell pellet. Subsequently cytocentrifuge preparations are made (50 μl of the cell suspension per preparation), which are fixed in methanol (30 min, 4°C). The cytocentrifuge preparations are subjected to the indirect TdT staining with a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit-Ig antiserum (Figures 5 and 9).

Double immunofluorescence staining for a cell surface membrane antigen and CyIg.

The cell suspension is incubated with the relevant antibody, which is directly or indirectly labeled with TRITC. Subsequently cytocentrifuge preparations are made. The cytocentrifuge preparations are fixed in acid ethanol (15 min, -20°C) and subjected to a direct CyIg staining, using a FITC-conjugated anti-human-Ig antiserum (Figure 10).

Double immunologic staining for SmIg and the MRBC receptor.

Fifty μl of the cell suspension is incubated with a FITC-conjugated anti-human-Ig antiserum. After the incubation and washings, the cell suspension is subjected to the M rosette test (see above). Subsequently the concentration of the cell suspension (MRBC plus nucleated cells) is adjusted to 2 x 10^6 cells per ml and cytocentrifuge preparations are made (50 μl of the cell suspension per preparation). The cytocentrifuge preparations are fixed in methanol (5 min, 4°C), mounted in glycerol, covered with a coverslip and sealed (Figure 11).

Triple immunologic staining

In the triple immunologic staining we use colloidal gold particles of 30 nm as the third label next to FITC and TRITC (125). Colloidal gold can be visualized in the same epi-illumination sys-

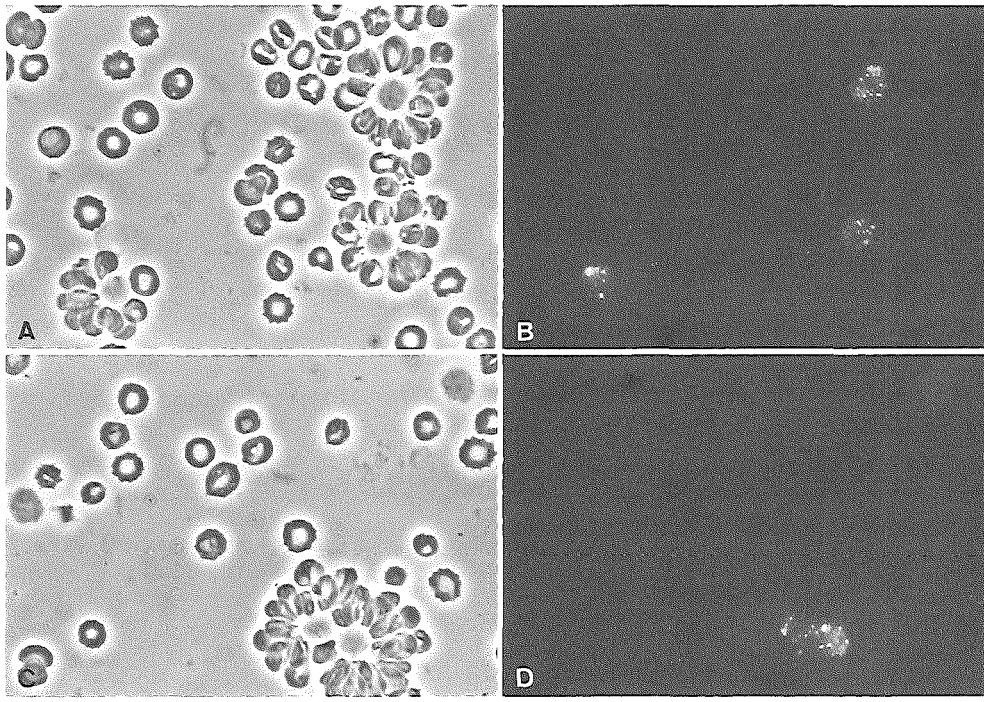


Figure 11. Double immunologic staining for Smλ and M rosette on MNC of PB from a CLL patient. **A** and **C**: phase contrast pictures; **B** and **D**: Smλ positive cells (FITC labeled). A and B represent the same field and C and D represent the same field. The M rosette forming cells also weakly expressed Smλ, while the Smλ negative cells did not form M rosettes.

tem, which is used for the evaluation of fluorescence (see Chapter 2.1). Although colloidal gold particles of 30 nm can easily be used as label for the detection of cytoplasmic markers of cells in tissue sections (141), this is more difficult when cytocentrifuge preparations are used. Therefore we use the colloidal gold technique for staining cell surface membrane markers. One example of a triple immunologic staining will be described.

Triple immunologic staining for the CD3 antigen, HLA-DR and TdT.

Fifty μl of the cell suspension is incubated with the CD3 McAb Leu-4 (Table 1), washed and subsequently incubated with 50 μl of an undiluted goat anti-mouse-Ig antiserum, conjugated with colloidal gold particles of 30 nm (GAM-G30; Janssen Pharmaceutica, Beerse, Belgium). Afterwards the cells are incubated with normal mouse serum (diluted 1 in 100) to block free antigen binding sites of the goat anti-mouse-Ig antiserum. Subsequently the cells are incubated with a TRITC-conjugated anti-HLA-DR McAb (Becton Dickinson). After the incubation and washings, 200 μl of PBS/0.5% BSA is added to the cell pellet. Finally, cytocentrifuge preparations are made, which are subjected to the indirect TdT staining using FITC as label (Figure 12).

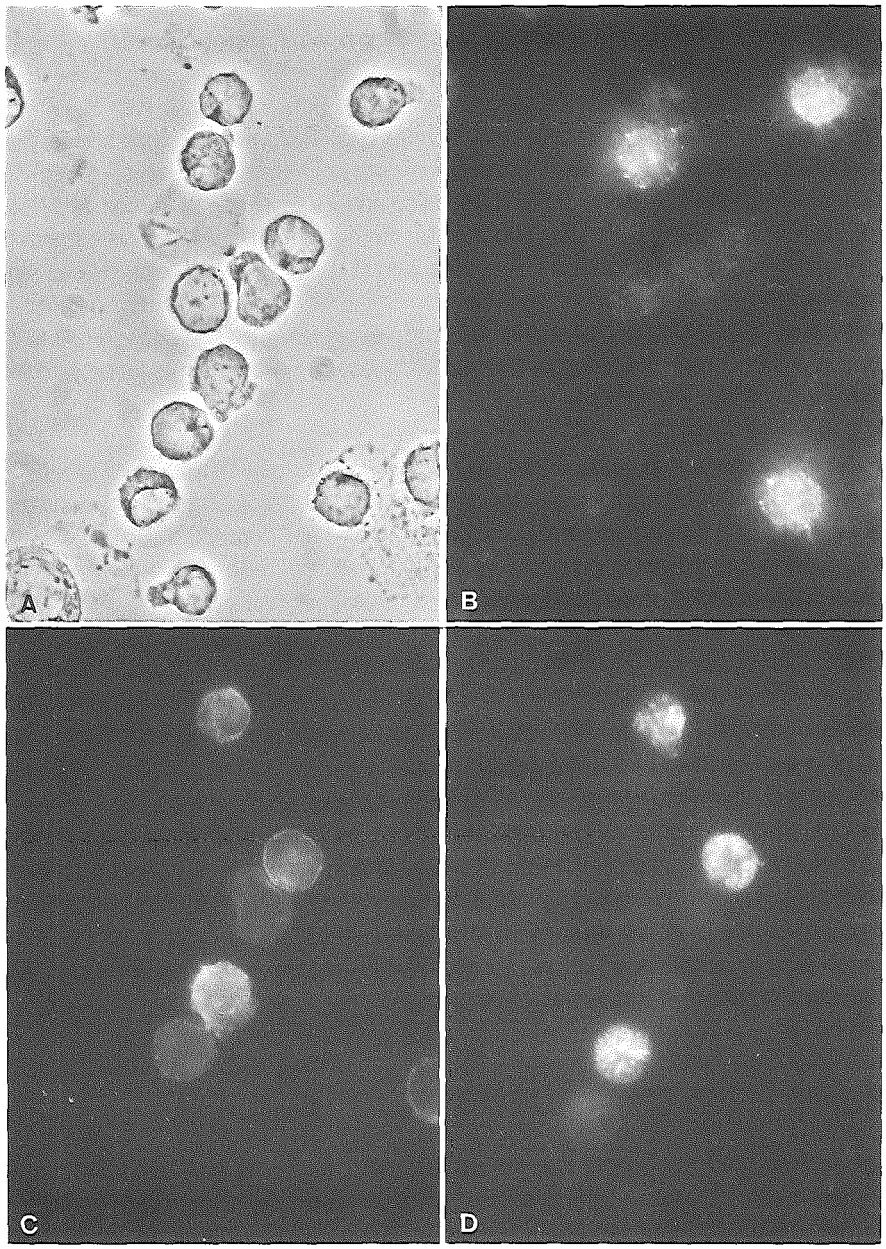


Figure 12. Triple immunologic staining for the CD3 antigen, HLA-DR and TdT on MNC of BM from a child with ALL in complete remission under maintenance treatment. **A:** phase contrast picture; **B:** CD3 (Leu-4) positive cells (colloidal gold labeled); **C:** HLA-DR positive cells (TRITC labeled); **D:** TdT positive cells (FITC labeled). The three TdT positive cells are also positive for HLA-DR, but negative for the CD3 (Leu-4) antigen. The former cells probably represent precursor B cells, while the CD3 positive cells represent mature T lymphocytes.

Microscopes

Evaluation of single and double immunologic stainings, using fluorochromes.

For the evaluation of fluorescence (FITC, TRITC and acridine orange), we use Zeiss Standard 16 microscopes (Carl Zeiss, Oberkochen, FRG), equipped with a IV FL Fluoreszenz-Auflichtkondensor (epi-illumination condensor) and phase contrast facilities. The epi-illumination system contains two filter combinations: Zeiss filter combination 14 (BP 510-560; FT 580; LP 590) for the evaluation of TRITC labelling and Zeiss filtercombination 19 (BP 485/20; FT 510; LP 515) combined with a KP 560 filter for the evaluation of FITC labeling and acridine orange staining. The microscopes are equipped with at least three Zeiss objectives : a neofluar 63/1.25 Oel Ph3 for evaluation of surface membrane staining, TdT staining, etc.; a planapochromat 40/1.0 Oel Ph3 for evaluation of strong expression of CyIg and a neofluar 16/0.40 Ph2 for evaluation of the rosette tests. These objectives are used in combination with the Zeiss wide-field oculars Kpl-W10x/18.

Evaluation of the triple immunologic staining, using colloidal gold as the third label.

We use two different microscopes for the evaluation of colloidal gold in combination with FITC and TRITC: a Zeiss Universal II (Carl Zeiss) and a Leitz Orthoplan (Ernst Leitz, Wetzlar, FRG). The Zeiss Universal II is equipped with a III RS Fluoreszenz- Auflichtkondensor, phase contrast facilities and the same filtercombinations, objectives and oculars as described for the Zeiss Standard 16 microscopes (see above). The Leitz Orthoplan is equipped with a Ploemopak 2 epi-illumination system, phase contrast facilities and the following two filtercombinations: Leitz filtercombination I2 (BP 450-490; RKP 510; LP 515) for the evaluation of FITC and Leitz filter combination N2 (BP 530-560; RKP 580; LP 580) for the evaluation of TRITC. The most frequently used objective of the Leitz microscope is the PL APO 63/1.40 Oel PHACO 4 in combination with the wide-field oculars Periplan GW 10xM.

For the evaluation of colloidal gold, we use a polarization filter combination in both microscopes, consisting of a polarizer for the excitation light, a beam-splitter and an analyzer, which extinguishes the reflected light that has the same polarization as the excitation beam. The objectives which are used for evaluation of colloidal gold should not contain phase contrast rings. According to our experience the best objective is the Zeiss Antiflex-Neofluar 63/1.25 Oel, which contains a rotatable quarter-wave plate (125).

IMMUNOLOGIC PHENOTYPES OF LEUKEMIAS AND NHL

Immunologic marker analysis can be applied not only to the characterization of normal hematopoietic cells, but also to the characterization of leukemias and NHL (Figure 2).

The maturation-arrest of the acute leukemias is localized in the immature differentiation stages (e.g. acute lymphoblastic leukemias (ALL) and acute myeloid leukemias (AML)). Chronic leukemias are the malignant counterparts of cells in more mature differentiation stages (e.g. chronic lymphocytic leukemias (CLL) and chronic myeloid leukemias (CML)). Generally, NHL have a mature immunologic phenotype.

Several differentiation stages may occur within one malignancy. This indicates that malig-

TABLE 2. Immunologic phenotypes of ALL.

	TdT	HLA-DR	CD19 and/ or CD22	CD10 (CALLA)	weak Cyμ	SmIg	pan-T cell markers (CD2,CyCD3,CD7)
null ALL	+	+	+	–	–	–	–
common ALL	+	+	+	+	–	–	–
pre-B-ALL	+	+	+	+	+	–	–
B-ALL	–	+	+	(+)[a]	–	+	–
T-ALL[b]	+	–[c]	–	–/+	–	–	+

a. Not always expressed.
b. Several subtypes of T-ALL can be recognized, based on their positivity for the various T cell markers (see ref. 22 and 143).
c. A few T-ALL may be HLA-DR positive (see ref. 58).

nant cells are capable of further maturation and that their maturation-arrest is not restricted to a single differentiation stage. This especially occurs in AML and NHL.

Lymphoid malignancies

ALL can be divided into at least five different types according to their immunologic phenotype : null ALL, common ALL, pre-B-ALL, B-ALL and T-ALL (6,7,19,22,23,142). These immunologic phenotypes are summarized in Table 2. T-ALL can be subdivided into several immature and more mature subtypes, as is indicated in Figures 2 and 3 (7,19,22,23,52,143). The morphological 'French American British' (FAB) classification of ALL recognizes three ALL types : L1, L2 and L3 (1,3). This FAB classification does not correlate well with the immunologic classification. L1 and L2 morphology can be found in most immunologic ALL types ; only L3 morphology seems to be restricted to the rarely occurring B-ALL.

Normally about 60% of the B cells express κ light chain, while about 30% express λ light chain; the normal κ/λ ratio varies from 0.8 to 2.0 (31). Since a B cell malignancy represents the clonal expansion of a single B cell, only one type of Ig light chain (either κ or λ) is expressed. Therefore, the κ/λ ratio is used for the detection of a B cell malignancy and may give an impression of the number of malignant cells (31,144). Several B cell malignancies can be distinguished by the use of additional B cell markers. B cell CLL (B-CLL) can be discriminated from other B cell malignancies by their weak expression of SmIg and positivity for the CD5 and CD6 antigen (117,118,120). On the other hand, the B cell prolymphocytic leukemia (B-PLL) strongly expresses SmIg and is generally negative for the CD5 and CD6 antigens (19,22). Hairy cell leukemia (HCL) is a B cell malignancy which expresses the "HCL-specific" B-ly-7 antigen as well as the CD11c and CD25 antigens (50,145,146), which are generally absent on B-CLL and B-PLL cells (147). The detailed immunologic phenotypes of B-CLL, HCL and B-PLL are summarized in Table 3.

Immunologic marker analysis of NHL does not result in a classification comparable to the detailed morphological classifications such as the Kiel classification (4,148). However, immunologic marker analysis is still valuable in the diagnosis of NHL (148). It is possible to

TABLE 3. Immunologic phenotypes of chronic B cell leukemias.

	CD5	CD6	CD11c	CD20	CD22	CD25	CD37	FMC7	SmIg	B-ly-7
B-CLL	+	+	–	±	±	–	+	–/±	±	–
HCL	–	–	+	+	+	+	+	+	+	+
B-PLL	–	–	–	+	+	–	+	+	++	–

Used symbols: –, no expression; ±, weak expression; +, normal expression; ++, strong expression.

determine whether a B cell or T cell malignancy is involved and to prove, in the case of a B cell malignancy, the clonality of the tumor by means of the κ/λ ratio (Figure 7) (31,144). In addition, more immunologic markers (especially B cell markers) have become available, which enable a better immunologic classification of NHL (17,18).

Myeloid malignancies

The various myeloid markers can be used for the classification of the myeloid malignancies, although they are not as discriminating as the lymphoid markers (Figures 2 and 4).

The morphological FAB classification of AML and the immunologic classification of AML are both based on differentiation characteristics (1-3,9,11,85,86). Although Figures 2 and 4 suggest that the FAB classification and immunologic classification of AML are similar, this is only true in the case of more mature AML (i.e. acute monocytic leukemia and acute promyelocytic leukemia) (11,24). AML often appear to consist of several subpopulations, which hampers the immunologic marker analysis of AML (24). Only double immunofluorescence stainings for the various myeloid markers allow the immunologic marker analysis of these subpopulations. CML cells express granulocytic markers such as CD15 and CD67 (Figure 4). When a CML transforms into a blastic crisis (BC), immunologic marker analysis can reveal whether it concerns a myeloid or lymphoid BC (Figure 9). The immunologic phenotypes of a myeloid BC are comparable to those of the various AML, while the phenotype of a lymphoid BC is generally similar to the phenotype of a common ALL or pre-B-ALL (22,149).

The true histiocytic NHL is very rare. By use of the various monocytic-macrophage markers (indicated in Figure 4 and Table 1) it is possible to characterize these NHL.

APPLICATION OF IMMUNOLOGIC MARKER ANALYSIS

Immunologic marker analysis of cells in normal hematopoietic differentiation stages

Immunologic marker analysis can be used for the characterization of hematopoietic cell populations, such as in patients with primary immunodeficiencies (150). It can also provide information concerning diseases, where the balance between various (sub)populations is dis-

TABLE 4. Application of immunologic marker analysis.

A. Immunologic marker analysis of cells in normal hematopoietic differentiation stages is important for:
- determination of percentages of T lymphocytes and CD4/CD8 ratios in cell samples from patients with diseases such as sarcoidosis, AIDS, etc.
- analysis of cell populations in BM, PB and lymphoid tissues from patients with (primary) immunodeficiencies.
- analysis of cultured cells, e.g. in the case of lymphocyte proliferation tests.

B. Immunologic marker analysis of leukemias and NHL is important for:
- classification of leukemias and NHL.
- recognition of subtypes of leukemias and NHL, which differ in clinical behaviour and therapy resistance.
- recognition of association between immunologic phenotypes of leukemias and NHL and chromosomal aberrations.
- detection of two malignancies in one patient.
- characterization of subpopulations within one malignancy.
- detection of low numbers of malignant cells.
- staging of NHL, especially TdT^+ T-NHL.

turbed (151,152). In patients with acquired immunodeficiency syndrome (AIDS) the CD4/CD8 ratio in PB becomes extremely low (153,154). In patients with active sarcoidosis the percentage T lymphocytes in BAL fluid is about 22% and sometimes up to 80% with high CD4/CD8 ratios, while in BAL fluid from normal controls, low percentages T lymphocytes are detected (155,156). The monitoring of percentages of T lymphocytes and CD4/CD8 ratios may be useful during follow-up of patients, who have undergone an organ transplantation (e.g. during anti-thymocyte globulin (ATG) treatment) (157,158) (Table 4).

In addition, immunologic marker analysis is used to determine the nature of cells in culture, such as in lymphocyte proliferation tests.

Immunologic marker analysis of leukemias and NHL

Immunologic marker analysis of leukemias and NHL enables a reproducible classification of these malignancies. By means of clinical trials it may be possible to recognize immunologic subtypes of leukemias and NHL, which differ in clinical behavior and therapy sensitivity (159). Therefore it is necessary to characterize these malignancies according to clinical, morphological and immunologic criteria, allowing adaptation of treatment protocols. Furthermore, immunologic marker analysis is important for the detection of associations between immunologic phenotypes and chromosome aberrations, to gain more insight in oncogenesis (160,161). In addition immunologic marker analysis allows the detection of two malignancies in one patient (162) and the characterization of subpopulations within one malignancy (163) (Table 4).

During maintenance therapy and even after termination of therapy it remains uncertain whether malignant cells are still present. The application of immunologic marker analysis can be used for the detection of low numbers of malignant cells, but is hampered by the presence of their normal counterparts (164). Therefore, cells positive for a certain marker or marker combination can only be regarded as malignant if these positive cells are present in higher

frequencies than normal or if they are detected outside their normal sites of occurrence. Since TdT positive cells are normally not detectable in CSF and since most ALL are positive for TdT, the TdT determination is valuable for the early detection of central nervous system leukemia (165). Another example of immunologic detection of low numbers of malignant cells is the double immunofluorescence staining for a T cell marker and TdT on cell samples from patients suffering from a TdT positive T cell malignancy (166-168). This approach is based on the fact that $CD1^+/TdT^+$, $CD3^+/TdT^+$, and $CD5^+/TdT^+$ cells normally occur in the thymus only (58,166,167). The application of these double stainings for analysis of BM and PB samples of T-ALL patients allows early detection of relapse (164,168) (for details see Chapters 5.3 and 5.4).

CONCLUSION

Immunologic marker analysis of normal hematopoietic cells and their malignant counterparts is an important tool for diagnostic use. It allows a precise analysis of leukocyte subpopulations in various diseases, such as immunodeficiencies. Moreover, it results in a reproducible classification of leukemias and NHL and allows, in several types of ALL and NHL, the detection of low numbers of malignant cells. Finally, it may be used not only for diagnostic purposes, but also for clinical research.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof.Dr. R. Benner for his continuous support, Mrs. A.P. Tio-Gillen and Dr. A.C. Knulst for critical reading of the manuscript, Mr. R. van den Beemd, Mrs. K. Benne, Mrs. W.M. Comans-Bitter, Mrs. E.G.M. Cristen, Mrs. A.A. van der Linde-Preesman, Mrs. A.F. Wierenga-Wolf, Mrs. A. Wijdenes-de Bresser and Mrs. I.L.M. Wolvers-Tettero for their technical assistance, Mr. C.J. van Dijk and Mr. T.M. van Os for their excellent photographic assistance and Mrs. J. de Goey-van Dooren for help in preparing the manuscript.

This work was supported by the Netherlands Cancer Foundation (Koningin Wilhelmina Fonds) and the Sophia Foundation for Medical Research (Sophia Children's Hospital, Rotterdam).

REFERENCES

1. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposals for the classification of the acute leukaemias. Br J Haematol 1976;33:451-8.
2. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7): a report of the French-American-British cooperative group. Ann Intern Med 1985;103:460-2.
3. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnick HR, Sultan C. Proposed revised criteria for the classification of acute leukemia: a report of the French-American-British cooperative group. Ann Intern Med 1985;103:626-9.
4. Lennert K, Mohri N, Stein H, Kaiserling E, Muller-Hermelink HK, eds. Malignant lymphomas: other than Hodgkin's disease. Berlin: Springer-Verlag, 1978.
5. The non-Hodgkin's lymphoma pathologic classification project. National Cancer Institute sponsored study of classifications of non-Hodgkin's lymphomas: summary and description of a working formulation for clinical usage. Cancer 1982;49:2112-35.
6. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic

cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
7. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
8. Tidman N, Janossy G, Bodger M, Granger S, Kung PC, Goldstein G. Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. Clin Exp Immunol 1981;45:457-67.
9. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557-63.
10. Hokland P, Ritz J. Schlossman SF, Nadler LM. Orderly expression of B cell antigens during the in vitro differentiation of nonmalignant human pre-B cells. J Immunol 1985;135:1746-51.
11. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 1985;75:746-53.
12. Stein H, Gerdes J, Mason DY. The normal and malignant germinal centre. Clin Haematol 1982;11:531-59.
13. Ling NR, Richardson PR. A critical appraisal of the direct antibody-rosette test for the detection of cell surface antigens. J Immunol Methods 1981;47:265-74.
14. Knapp W, ed. Leukemia markers. London: Academic Press, 1981.
15. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Human leucocyte differentiation antigens detected by monoclonal antibodies. Berlin: Springer-Verlag, 1984.
16. Reinherz EL, Haynes BF, Nadler LM, Bernstein ID, eds. Leucocyte typing II. Volume 1: Human T lymphocytes; Volume 2: Human B lymphocytes; Volume 3: Human myeloid and hematopoietic cells. Berlin: Springer-Verlag, 1986.
17. McMichael AJ, Beverley PCL, Gilks W, Horton M, Mason DY, Cobbold S, Gotch FM, Ling N, Milstein C, Waldmann H, Crumpton MJ, Hogg N, MacLennan ICM, Spiegelhalter D, eds. Leucocyte typing III: white cell differentiation antigens. Oxford: Oxford University Press, 1987.
18. Knapp W, Dörken B, Rieber EP, Stein H, Gilks WR, Schmidt RE, Von dem Borne AEGKr, eds. Leucocyte Typing IV: white cell differentiation antigens. Oxford: Oxford University Press, 1989.
19. Foon KA, Schroff RW, Gale RP. Surface markers on leukemia and lymphoma cells: recent advances. Blood 1982;60:1-19.
20. Van der Valk P, Jansen J, Daha MR, Meijer CJLM. Characterization of B-cell non-Hodgkin's lymphomas. A study using a panel of monoclonal and heterologous antibodies. Virchows Arch 1983;401:289-305.
21. Chan LC, Pegram SM, Greaves MF. Contribution of immunophenotype to the classification and differential diagnosis of acute leukaemia. Lancet 1985;i:475-9.
22. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
23. Greaves MF. Differentiation-linked leukemogenesis in lymphocytes. Science 1986;234:697-704.
24. Drexler HG. Classification of acute myeloid leukemias - a comparison of FAB and immunophenotyping. Leukemia 1987;1:697-705.
25. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
26. Goldschneider I, Gregoire KE, Barton RW, Bollum FJ. Demonstration of terminal deoxynucleotidyl transferase in thymocytes by immunofluorescence. Proc Natl Acad Sci USA 1977;74:734-8.
27. Raff MC, Megson M, Owen JJT, Cooper MD. Early production of intracellular IgM by B-lymphocyte precursors in mouse. Nature 1976;259:224-6.
28. Burrows P, LeJeune M, Kearney JF. Evidence that murine pre-B cells synthesise μ heavy chains but no light chains. Nature 1979;280:838-40.
29. Vogler LB, Crist WM, Bockman DE, Pearl ER, Lawton AR, Cooper MD. Pre-B-cell leukemia: a new phenotype of childhood lymphoblastic leukemia. New Engl J Med 1978;289:872-8.
30. Vogler LB, Crist WM, Sarrif AM, Pullen DJ, Bartolucci AA, Falletta JM, Dowell B, Humphrey GB, Blackstock R, Van Eys J, Metzgar RS, Cooper MD. An analysis of clinical and laboratory features of acute lymphocytic leukemias with emphasis on 35 children with pre-B leukemia. Blood 1981;58:135-40.
31. Teodorescu M, Mayer EP. Surface immunoglobulin in immunoproliferative diseases. Ann Clin Lab Sci 1978;8:353-65.
32. Vessière-Louveaux FMYR, Hijmans W, Schuit HRE. Multiple heavy chain isotypes on the membrane of the small B lymphocytes in human blood. Clin Exp Immunol 1981;43:149-56.
33. Hijmans W, Schuit HRE, Klein F. An immunofluorescence procedure for the detection of intracellular

immunoglobulins. Clin Exp Immunol 1969;4:457-72.
34. Nadler LM, Anderson KC, Marti G, Bates M, Park E, Daley JF, Schlossman SF. B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated and malignant B lymphocytes. J Immunol 1983;131:244-50.
35. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
36. Boué DR, LeBien TW. Expression and structure of CD22 in acute leukemia. Blood 1988;71:1480-6.
37. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF. A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 1980;283:583-5.
38. Knapp W, Majdic O, Bettelheim P, Liszka K. VIL-A1, a monoclonal antibody reactive with common acute lymphatic leukemia cells. Leuk Res 1982;6:137-47.
39. LeBien T, Kersey J, Nakazawa S, Minato K, Minowada J. Analysis of human leukemia/lymphoma cell lines with monoclonal antibodies BA-1, BA-2 and BA-3. Leuk Res 1982;6:299-305.
40. McKenzie IFC, Zola H. Monoclonal antibodies to B cells. Immunol Today 1983;4:10-5.
41. Zola H. The surface antigens of human B lymphocytes. Immunol Today 1987;8:308-15.
42. Kersey JH, LeBien TW, Abramson CS, Newman R, Sutherland R, Greaves M. p24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. J Exp Med 1981;153:726-31.
43. Ash RC, Jansen J, Kersey JH, LeBien TW, Zanjani ED. Normal human pluripotential and committed hematopoietic progenitors do not express the p24 antigen detected by monoclonal antibody BA-2: implications for immunotherapy of lymphocytic leukemia. Blood 1982;60:1310-6.
44. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. J Immunol 1980;125:1678-85.
45. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Expression of cell surface markers after human B lymphocyte activation. Proc Natl Acad Sci USA 1981;78:3848-52.
46. Nadler LM, Stashenko P, Hardy R, Van Agthoven A, Terhorst C, Schlossman SF. Characterization of a human B cell-specific antigen (B2) distinct from B1. J Immunol 1981;126:1941-7.
47. Forster HK, Gudat FG, Girard MF, Albrecht R, Schmidt J, Ludwig C, Obrecht JP. Monoclonal antibody against a membrane antigen characterizing leukemic human B-lymphocytes. Cancer Res 1982;42:1927-34.
48. Brooks DA, Beckman IGR, Bradley J, McNamara PJ, Thomas ME, Zola H. Human lymphocyte markers defined by antibodies derived from somatic cell hybrids. IV. A monoclonal antibody reacting specifically with a subpopulation of human B lymphocytes. J Immunol 1981;126:1373-7.
49. Catovsky D, Cherchi M, Brooks D, Bradley J, Zola H. Heterogeneity of B-cell leukemias demonstrated by the monoclonal antibody FMC7. Blood 1981;58:406-8.
50. Visser L, Shaw A, Slupsky J, Vos H, Poppema S. Monoclonal antibodies reactive with hairy cell leukemia. Blood 1989;74:320-5.
51. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA 1980;77:1588-92.
52. Janossy G, Prentice HG. T cell subpopulations, monoclonal antibodies and their therapeutic applications. Clin Haematol 1982;11:631-60.
53. Haynes BF, Mann DL, Hemler ME, Schroer JA, Shelhamer JH, Eisenbarth GS, Strominger JL, Thomas CA, Mostowski HS, Fauci AS. Characterization of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans. Proc Natl Acad Sci USA 1980;77:2914-8.
54. Vodinelich L, Tax W, Bai Y, Pegram S, Capel P, Greaves MF. A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 1983;62:1108-13.
55. Sutherland DR, Rudd CE, Greaves MF. Isolation and characterization of a human T lymphocyte-associated glycoprotein (gp40). J Immunol 1984;133:327-33.
56. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J Immunol 1981;126:2117-22.
57. Verbi W, Greaves MF, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G. Monoclonal antibodies OKT11 and OKT11A have pan-T reactivity and block sheep erythrocyte "receptors". Eur J Immunol 1982;12:81-6.

58. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
59. Moore AL, Zusman J. Conditions enhancing the percentage and size of E rosettes formed by T lymphocytes. J Immunol Methods 1978;23:275-84.
60. Campana D, Janossy G, Bofill M, Trejdosiewicz LK, Ma D, Hoffbrand AV, Mason DY, Lebacq A-M, Forster HK. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985;134:1524-30.
61. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
62. Engleman EG, Warnke R, Fox RI, Dilley J, Benike CJ, Levy R. Studies of a human T lymphocyte antigen recognized by a monoclonal antibody. Proc Natl Acad Sci USA 1981;78:1791-5.
63. Martin PJ, Hansen JA, Siadak AW, Nowinski RC. Monoclonal antibodies recognizing normal human T lymphocytes and malignant human B lymphocytes: a comparative study. J Immunol 1981;127:1920-3.
64. Engleman EG, Benike CJ, Glickman E, Evans RL. Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man. J Exp Med 1981;153:193-8.
65. Ledbetter JA, Evans RL, Lipinski M, Cunningham-Rundles C, Good RA, Herzenberg LA. Evolutionary conservation of surface molecules that distinguish T lymphocyte helper/inducer and cytotoxic/suppressor subpopulations in mouse and man. J Exp Med 1981;153:310-23.
66. Borst J, Prendiville MA, Terhorst C. Complexity of the human T lymphocyte-specific cell surface antigen T3. J Immunol 1982;128:1560-5.
67. Reiter C. Cluster report: CD6. In: Knapp W, Dörken B, Gilks WR, Rieber EP, Schmidt RE, Stein H, Von dem Borne AEGKr. Leucocyte Typing IV. White cell differentiation antigens. Oxford: Oxford University Press, 1989:339.
68. Lerch PG, Van de Rijn M, Schrier P, Terhorst C. Biochemical comparison of the T6 antigen and HLA-A,B antigens. Hum Immunol 1983;6:13-30.
69. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function. J Exp Med 1983;157:705-19.
70. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The δ- and ϵ-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature 1984;312:455-8.
71. Owen MJ. T-cell receptor companions. Nature 1984;312:406.
72. Hood L, Kronenberg M, Hunkapiller T. T cell antigen receptors and the immunoglobulin supergene family. Cell 1985;40:225-9.
73. Acuto O, Reinherz EL. The human T-cell receptor: structure and function. New Engl J Med 1985;312:1100-11.
74. Marrack P, Kappler J. The T cell and its receptor. Sci Am 1986;254-2:36-45.
75. Weiss A, Imboden J, Shoback D, Stobo J. Role of T3 surface molecules in human T-cell activation: T3-dependent activation results in an increase in cytoplasmic free calcium. Proc Natl Acad Sci USA 1984;81:4169-73.
76. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402.
77. Strominger JL. Developmental biology of T cell receptors. Science 1989;224:943-50.
78. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 1985;135:1922-8.
79. Borst J, Van Dongen JJM, De Vries E, Comans-Bitter WM, Van Tol MJD, Vossen JM, Kurrle R. BMA031, a monoclonal antibody suited to identify the T cell receptor $\alpha\beta$/CD3 complex on viable human T lymphocytes in normal and disease states. Hum Immunol 1990, in press.
80. Band H, Hochstenbach F, McLean J, Hata S, Krangel MS, Brenner MB. Immunochemical proof that a novel rearranging gene encodes the T cell receptor δ subunit. Science 1987;238:682-4.
81. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.

82. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986;136:4480-6.
83. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 1983;131:1789-96.
84. Hogg N. The structure and function of Fc receptors. Immunol Today 1988;9:185-7.
85. Griffin JD, Ritz J, Nadler LM, Schlossman SF. Expression of myeloid differentiation antigens on normal and malignant myeloid cells. J Clin Invest 1981;68:932-41.
86. Griffin JD, Larcom P, Schlossman SF. Use of surface markers to identify a subset of acute myelomonocytic leukemia cells with progenitor cell properties. Blood 1983;62:1300-3.
87. Majdic O, Bettelheim P, Stockinger H, Aberer W, Liszka K, Lutz D, Knapp W. M2, a novel myelomonocytic cell surface antigen and its distribution on leukemic cells. Int J Cancer 1984;33:617-23.
88. Van der Schoot CE, Daams GM, Pinkster J, Vet R, Von dem Borne AEGKr. Monoclonal antibodies against myeloperoxidase are valuable immunological reagents for the diagnosis of acute myeloid leukaemia. Br J Haematol 1990;74:173-8.
89. Brooks DA, Zola H, McNamara PJ, Bradley J, Bradstock KF, Hancock WW, Atkins RC. Membrane antigens of human cells of the monocyte/macrophage lineage studied with monoclonal antibodies. Pathol 1983;15:45-52.
90. Hancock WW, Zola H, Atkins RC. Antigenic heterogeneity of human mononuclear phagocytes: immunohistologic analysis using monoclonal antibodies. Blood 1983;62:1271-9.
91. Hance AJ, Douches S, Winchester RJ, Ferrans VJ, Crystal RG. Characterization of mononuclear phagocyte subpopulations in the human lung by using monoclonal antibodies: changes in alveolar macrophage phenotype associated with pulmonary sarcoidosis. J Immunol 1985;134:284-92.
92. Ugolini V, Nunez G, Smith RG, Stastny P, Capra JD. Initial characterization of monoclonal antibodies against human monocytes. Proc Natl Acad Sci USA 1980;77:6764-8.
93. Radzun HJ, Kreipe H, Zavazava N, Hansmann HL, Parwaresch MR. Diversity of the human monocyte/macrophage system as detected by monoclonal antibodies. J Leukocyte Biol 1988;43:41-50.
94. Munro CS, Campbell DA, Collings LA, Poulter LW. Monoclonal antibodies distinguish macrophages and epithelioid cells in sarcoidosis and leprosy. Clin Exp Immunol 1987;68:282-7.
95. Majdic O, Liszka K, Lutz D, Knapp W. Myeloid differentiation antigen defined by a monoclonal antibody. Blood 1981;58:1127-33.
96. Hanjan SNS, Kearney JF, Cooper MD. A monoclonal antibody (MMA) that identifies a differentiation antigen on human myelomonocytic cells. Clin Immunol Immunopathol 1982;23:172-88.
97. Villeval JL, Cramer P, Lemoine F, Henri A, Bettaieb A, Bernaudin F, Beuzard Y, Berger R, Flandrin G, Breton-Gorius J, Vainchenker W. Phenotype of early erythroblastic leukemias. Blood 1986;68:1167-74.
98. Andersson LC, Gahmberg CG, Teerenhovi L, Vuopio P. Glycophorin A as a cell surface marker of early erythroid differentiation in acute leukemia. Int J Cancer 1979;23:717-20.
99. Liszka K, Majdic O, Bettelheim P, Knapp W. Glycophorin A expression in malignant hematopoiesis. Am J Hematol 1983;15:219-26.
100. Loken MR, Shah VO, Dattilio KL, Civin CI. Flow cytometric analysis of human bone marrow: I. Normal erythroid development. Blood 1987;69:255-63.
101. San Miguel JF, Gonzalez M, Cañizio MC, Ojeda E, Orfao A, Caballero MD, Moro MJ, Fisac P, Lopez Borrasca A. Leukemias with megakaryoblastic involvement: clinical, hematologic, and immunologic characteristics. Blood 1988;72:402-7.
102. Clemetson KJ, Lüscher EF. Membrane glycoprotein abnormalities in pathological platelets. Biochim Biophys Acta 1988;947:53-73.
103. Kunicki TJ. Platelet membrane glycoproteins and their function: an overview. Blut 1989;59:30-4.
104. Breton-Gorius J, Villeval JL, Kieffer N, Mitjavila MT, Guichard J, Vainchenker W. Limits of phenotypic markers for the diagnosis of megakaryoblastic leukemia. Blood Cells 1989;15:259-77.
105. Reinherz EL, Kung PC, Pesando JM, Ritz J, Goldstein G, Schlossman SF. Ia determinants on human T-cell subsets defined by monoclonal antibody. Activation stimuli required for expression. J Exp Med 1979;150:1472-82.
106. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:1472-82.

107. Falkenburg JHF, Jansen J, Van der Vaart-Duinkerken N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ. Polymorphic and monomorphic HLA-DR determinants on human hematopoietic progenitor cells. Blood 1984;63:1125-32.
108. Hercend T, Ritz J, Schlossman SF, Reinherz EL. Comparative expression of T9, T10 and Ia antigens on activated human T cell subsets. Hum Immunol 1981;3:247-59.
109. Sutherland R, Delia D, Schneider C, Newman R, Kemshead J, Greaves M. Ubiquitous cell-surface glycoprotein on tumor cells is proliferation-associated receptor for transferrin. Proc Natl Acad Sci USA 1981;78:4515-9.
110. Van de Rijn M, Geurts van Kessel AHM, Kroezen V, Van Agthoven AJ, Verstijnen K, Terhorst C, Hilgers J. Localization of a gene controlling the expression of the human transferrin receptor to the region q12→qter of chromosome 3. Cytogenet Cell Genet 1983;36:525-31.
111. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D, Birnie GD. A novel monoclonal antibody BI-3C5 recognises myeloblasts and non-B non-T lymphoblasts in acute leukaemias and CGL blast crises, and reacts with immature cells in normal bone marrow. Leuk Res 1985;9:1-9.
112. Huizinga TWJ, Roos D, Von dem Borne AEGKr. Neutrophil Fc-γ receptors: a two-way bridge in the immune system. Blood 1990;75:1211-4.
113. Sanchez-Madrid F, Nagy JA, Robbins E, Simon P, Springer TA. A human leukocyte differentiation antigen family with distinct α-subunits and a common β-subunit: the lymphocyte function-associated antigen (LFA-1), the C3bi complement receptor (OKM1/Mac-1) and the p150,95 molecule. J Exp Med 1983;158:1785-83.
114. Arnaout MA. Structure and function of the leukocyte adhesion molecules CD11/CD18. Blood 1990;75:1037-50.
115. Fithian E, Kung P, Goldstein G, Rubenfeld M, Fenoglio C, Edelson R. Reactivity of Langerhans cells with hybridoma antibody. Proc Natl Acad Sci USA 1981;78:2541-4.
116. Nicholson JKA, Cross GD, Callaway CS, McDougal JS. In vitro infection of human monocytes with human T lymphotropic virus type III/lymphadenopathy-associated virus (HTLV-III/LAV). J Immunol 1986;137:323-9.
117. Royston I, Majda JA, Baird SM, Meserve BL, Griffiths JC. Human T cell antigens defined by monoclonal antibodies: the 65,000-dalton antigen of T cells (T65) is also found on chronic lymphocytic leukemia cells bearing surface immunoglobulin. J Immunol 1980;125:725-31.
118. Caligaris-Cappio F, Gobbi M, Bofill M, Janossy G. Infrequent normal B lymphocytes express features of B-chronic lymphocytic leukemia. J Exp Med 1982;155:623-8.
119. Burns BF, Warnke RA, Doggett RS, Rouse RV. Expression of a T-cell antigen (Leu-1) by B-cell lymphomas. Am J Pathol 1983;113:165-71.
120. Kamoun M, Kadin ME, Martin PJ, Nettleton J, Hansen JA. A novel human T cell antigen preferentially expressed on mature T cells and shared by both well and poorly differentiated B cell leukemias and lymphomas. J Immunol 1981;127:987-91.
121. McGraw DJ, Kurec AS, Davey FR. Mouse erythrocyte formation. A marker for resting B lymphocytes. Am J Clin Pathol 1981;77:177-83.
122. Neudorf SML, LeBien TW, Kersey JH. Characterization of thymocytes expressing the common acute lymphoblastic leukemia antigen. Leuk Res 1984;8:173-9.
123. Braun MP, Marin PJ, Ledbetter JA, Hansen JA. Granulocytes and cultured human fibroblasts express common acute lymphoblastic leukemia-associated antigens. Blood 1983;61:718-25.
124. Abramson CS, Kersey JH, LeBien TW. A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. J Immunol 1981;126:83-8.
125. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM. De Josselin de Jong J. Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.
126. Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY. Immunoenzymatic staining of haematological samples with monoclonal antibodies. Br J Haematol 1983;55:395-410.
127. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Isolation of mononuclear cells by one centrifugation, and of granulocytes by combining centrifugation and sedimentation at 1 g. Scand J Clin Lab Invest 1968;21:77-89.
128. Titus JA, Haugland R, Sharrow SO, Segal DM. Texas red, a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. J Immunol

Methods 1982;50:193-204.
129. Oi VT, Glazer AN, Stryer L. Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. J Cell Biol 1982;93:981-6.
130. Janossy G, Campana D. Immunofluorescence studies in leukaemia diagnosis. In: P. Beverley, ed. Methods in haematology : monoclonal antibodies. Edinburgh: Churchill Livingstone, 1985:97-131.
131. Mason DY, Stein H, Naiem M, Abdulaziz Z. Immunohistological analysis of human lymphoid tissue by double immunoenzymatic labelling. J Cancer Res Clin Oncol 1981;101:13-22.
132. Feller AC, Parwaresch MR. Simultaneous enzyme-immunocytochemical detection of antigens in monocellular specimens with monoclonal antibodies. J Immunol Methods 1983;63:273-9.
133. Van Rooijen N, Kors N. Double immunocytochemical staining in the study of antibody-producing cells in vivo. Combined detection of antigen specificity (anti-TNP) and (sub)class of intracellular antibodies. J Histochem Cytochem 1985;33:175-8.
134. Van Rooijen N, Kors N, Claassen E, Boorsma DM. Binding of different antigen-enzyme and antibody-enzyme conjugates by intracellular antibodies in cytoplasm and Golgi complex of plasma cells. A double immunocytochemical study. Histochem 1985;83:61-3.
135. Norton AJ, Isaacson PG. Lymphoma phenotyping in formalin-fixed and paraffin wax-embedded tissue: I. Range of antibodies and staining patterns. Histopathol 1989;14:437-46.
136. Foon KA, Gale RP, Todd III RF. Immunologic approaches to the classification of lymphomas and lymphoid leukemias. In: Bennett JM, Foon KA, eds. Immunologic approaches to the classification and management of lymphomas and leukemias. Dordrecht: Kluwer Academic Publishers, 1988:1-30.
137. Mason DY, Erber WN, Falini B, Stein H, Gatter KC. Immuno-enzymatic labelling of haematological sample with monoclonal antibodies. In: Beverley PCL, ed. Methods in haematology: monoclonal antibodies. Edinburgh: Churchill Livingstone, 1986:145-81.
138. Johnson GD, De C. Nogueira Araujo GM. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 1981;43:349-50.
139. Guesdon JL, Ternynck T, Avrameas S. The use of avidin-biotin interaction in immunoenzymatic techniques. J Histochem Cytochem 1979;27:1131-9.
140. Simmonds RG, Smith W, Marsden H. 3-Phenylazo-4-hydroxyphenylisothiocyanates: reagents for efficient haptenation of immunoglobulin and other carrier molecules. J Immunol Methods 1982;54:23-30.
141. De Mey J. Colloidal gold probes in immunocytochemistry. In: Polak JM, Van Noorden S, eds. Immunocytochemistry: Practical applications in pathology and biology. Bristol: Wright PSG, 1983:82-112.
142. Van der Reijden HJ, Van Wering ER, Van de Rijn JM, Melief CJM, Van't Veer MB, Behrendt H, Von dem Borne AEGKr. Immunological typing of acute lymphoblastic leukaemia. Scand J Haematol 1983;30:356-66.
143. Greaves MF, Rao J, Hariri G, Verbi W, Catovsky D, Kung P, Goldstein G. Phenotypic heterogeneity and cellular origins of T cell malignancies. Leuk Res 1981;5:281-99.
144. Smith BR, Weinberg DS, Robert NJ, Towle M, Luther E, Pinkus GS, Ault KA. Circulating monoclonal B lymphocytes in non-Hodgkin's lymphoma. New Engl J Med 1984;311:1476-81.
145. Schwarting R, Stein H, Wang CY. The monoclonal antibodies αS-HCL 1 (αLeu-14) and αS-HCL 3 (αLeu-M5) allow the diagnosis of hairy cell leukemia. Blood 1985;65:974-83.
146. Korsmeyer SJ, Greene WC, Cossman J, Hsu S-M, Jensen JP, Neckers LM, Marshall SL, Bakhshi A, Depper JM, Leonard WJ, Jaffe ES, Waldmann TA. Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia. Proc Natl Acad Sci USA 1983;80:4522-6.
147. Caligaris-Cappio F, Janossy G. Surface markers in chronic lymphoid leukemias of B cell type. Semin Hematol 1985;22:1-12.
148. Schuurman H-J, Van Baarlen J, Huppes W, Lam BW, Verdonck LF, Van Unnik JAM. Immunophenotyping of non-Hodgkin's lymphoma: lack of correlation between immunophenotype and cell morphology. Am J Pathol 1987;129:140-51.
149. Canellos GP, Griffin JD. Chronic granulocytic leukemia: the heterogeneity of stem cell differentiation within a single disease entity. Semin Oncol 1985;12:281-9.
150. Davies EG, Levinsky RJ, Butler M, Thomas RM, Linch DC. Lymphocyte subpopulations in primary immunodeficiency disorders. Arch Dis Child 1983;58:346-51.
151. Bach MA, Bach JF. The use of monoclonal anti-T cell antibodies to study T cell imbalances in human diseases. Clin Exp Immunol 1981;45:449-56.
152. Goodwin JS. OKT3, OKT4, and all that. JAMA 1981;246:947-8.

153. Gottlieb MS, Schroff R, Schanker HM, Weisman JD, Thim Fan P, Wolf RA, Saxon A. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men. New Engl J Med 1981;305:1425-31.
154. Gottlieb MS, Groopman JE, Weinstein WM, Fahey JL, Detels R. The acquired immunodeficiency syndrome. Ann Int Med 1983;99:28-20.
155. Crystal RG, Roberts WC, Hunninghake GW, Gadek JE, Fulmer JD, Line BR. Pulmonary sarcoidosis: a disease characterized and perpetuated by activated lung T-lymphocytes. Ann Int Med 1981;94:73-94.
156. Crystal RG, Bitterman PB, Rennard SI, Hance AJ, Keogh BA. Interstitial lung diseases of unknown cause. Disorders characterized by chronic inflammation of the lower respiratory tract. New Engl J Med 1984;310:235-44.
157. Cosimi AB, Colvin RB, Burton RC, Rubin RH, Goldstein G, Kung PC, Hansen WP, Delmonico FL, Russell PS. Use of monoclonal antibodies to T-cell subsets for immunologic monitoring and treatment in recipients of renal allografts. New Engl J Med 1981;305:308-14.
158. Shen SY, Weir MR, Kosenko A, Revie DR, Ordonez JV, Dagher FJ, Chretien P, Sadler JH. Reevaluation of T cell subset monitoring in cyclosporine-treated renal allograft recipients. Transplantation 1985;40:620-3.
159. Greaves MF, Janossy G, Peto J, Kay H. Immunologically defined subclasses of acute lymphoblastic leukaemia in children: their relationship to presentation features and prognosis. Br J Haematol 1981;48:179-97.
160. Williams DL, Look AT, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S. New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia. Cell 1984;36:101-9.
161. Uckun FM, Gajl-Peczalska KJ, Provisor AJ, Heerema NA. Immunophenotype-karyotype associations in human acute lymphoblastic leukemia. Blood 1989;73:271-80.
162. Van Dongen JJM, Hooijkaas H, Michiels JJ, Grosveld G, De Klein A, Van der Kwast ThH, Prins MEF, Abels J, Hagemeijer A. Richter's syndrome with different immunoglobulin light chains and different heavy chain gene rearrangements. Blood 1984;64:571-5.
163. Van Dongen JJM, Hooijkaas H, Hagemeijer A, Hählen K, Van Zanen GE, Lansdorp PM. Characterization and monitoring of the malignant cells in the blastic and chronic phase of chronic myeloid leukemia. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1984;31:711-4.
164. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986:113-33.
165. Hooijkaas H, Van Dongen JJM, Hählen K, Van Zanen GE. Immunological characterisation of cells in cerebrospinal fluid from patients with lymphoid malignancies. Lancet 1984;i:518-9.
166. Bradstock KF, Janossy G, Hoffbrand AV, Ganeshagury K, Llewellin P, Prentice HG, Bollum FJ. Immunofluorescent and biochemical studies of terminal deoxynucleotidyl transferase in treated acute leukaemia. Br J Haematol 1981;47:121-31.
167. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res 1981;5:301-9.
168. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: Martinus Nijhoff Publishers, 1984:67-81.

CHAPTER 2.4

TRIPLE IMMUNOLOGIC STAINING WITH COLLOIDAL GOLD, FLUORESCEIN AND RHODAMINE LABELS*

J.J.M. van Dongen[1], H. Hooijkaas[1], W.M. Comans-Bitter[1,2], K. Benne[1], T.M. van Os[1] and J. de Josselin de Jong[3]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
2. Department of Pediatrics, Sophia Children's Hospital, Rotterdam;
3. Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands.

SUMMARY

Colloidal gold particles are detectable by light microscopy with polarized light in the same epi-illumination system as for fluorescence microscopy. Colloidal gold particles can thus be used in combination with a fluorochrome for the combined immunologic detection of surface membrane and cytoplasmic markers. We analyzed human bone marrow cells by a triple immunologic staining for 3 different markers, using colloidal gold, fluorescein and rhodamine as labels. Our results demonstrate that such a triple immunologic staining provides a powerful tool for study of the heterogeneity of small cell populations.

INTRODUCTION

In microscopy, immunologic staining techniques are widely used for both research and diagnostic purposes, especially for the detection of surface membrane and cytoplasmic markers. The antibodies used can be conjugated with fluorochromes such as fluorescein isothiocyanate (FITC) or tetramethylrhodamine isothiocyanate (TRITC), or with enzymes such as peroxidase (PO) or alkaline phosphatase(AP). For simultaneous detection of different markers, a double staining technique can be used, e.g. FITC in combination with TRITC (1) or PO in combination with AP (2,3).

Double staining techniques provide additional information about marker distribution within a cell population that cannot be obtained by 2 separate stainings of individual samples. For instance, the combined detection of kappa and lambda immunoglobulin (Ig) light chains on different cells proves the B cell character of these cells, while their presence on the same cell indicates Fc receptor binding of autologous Ig, e.g. by monocytes (4). Another example of the useful application of double staining is the simultaneous detection of a T cell marker and the

* *Published in: J Immunol Methods 1985;80:1-6. Updated for CD codes.*

nuclear enzyme terminal deoxynucleotidyl transferase (TdT) on single cells. Since T cell marker$^+$/TdT$^+$ cells normally occur only in the thymus, the presence of cells with the T cell marker$^+$/TdT$^+$ phenotype in extra-thymic locations for indicative of T cell acute lymphoblastic leukemia (T-ALL) or TdT-positive T cell non-Hodgkin lymphoma (5,6).

For the identification of small cell populations, it may be mandatory to use a triple staining technique for detection of 3 different markers at the single cell level. In microscopy it is difficult to combine FITC and TRITC with a third fluorochrome, e.g. 4-acetamido-4´isothiocyanato stilbene-2,2´-disulphonic acid (SITS) (7), Texas Red (8) or phycoerythrin (9), because of marked fading or a considerable overlap in excitation and emission spectra (10).

Recently a technique was described which allows the detection of colloidal gold particles in light microscopy by epi-illumination with polarized light (11). In theory, colloidal gold particles are a suitable label for combining with fluorochromes. We adapted this technique for triple staining with colloidal gold, FITC and TRITC.

MATERIALS AND METHODS

Preparation of cell suspension

Bone marrow (BM) cells from children treated for non-T-ALL and from healthy adult volunteers were obtained by needle aspiration. Mononuclear cells (MNC) were isolated by Ficoll-Paque (density 1.077 g/ml; Pharmacia, Uppsala) density centrifugation (12). The MNC were washed twice. These and all subsequent washings of cell suspensions were performed with phosphate-buffered saline (PBS) (300 mosmol; pH 7.8), supplemented with 1% bovine serum albumin (BSA; Organon Teknika, Oss) and 0.05% sodium azide. The cell concentration was adjusted to 10^7 cells per ml.

Triple staining technique

We performed triple staining for the detection of the T cell marker CD7 (13), a non-polymorphic HLA-DR antigen (14) and the nuclear enzyme TdT. Fifty microliters of the cell suspension were incubated with 50 μl of the optimally titrated monoclonal antibody (McAb) CD7 (3A1) (American Type Culture Collection, Rockville, MD) at room temperature (RT). After 30 min the cells were washed twice and incubated (30 min, RT) with 50 μl undiluted goat anti-mouse Ig antiserum, conjugated with colloidal gold particles of 30 nm (G30; Janssen Pharmaceutica, Beerse). The cells were washed twice and subsequently incubated (30 min, RT) with normal mouse serum (diluted 1 in 100 in PBS) to block free antigen binding sites of the goat anti-mouse Ig serum. The cells were washed twice and incubated (30 min, RT) with a TRITC-conjugated anti-HLA-DR McAb (Becton Dickinson, Sunnyvale, CA). After 2 washings the cell suspension was adjusted to 2×10^6 cells per ml. Fifty microliters of this cell suspension were centrifuged on slides in a cytocentrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands). The cytocentrifuge preparations were air dried during 15 min and the location of the cells was marked by encircling with a glass pencil. The preparations were fixed in methanol (30 min, 4°C) and washed in PBS for 15 min. The slides were dried and incubated with 15 μl of a rabbit anti-TdT antiserum (Bethesda Research Laboratory, Gaithersburg, MD) in a moist chamber (30 min, RT). The slides were then washed in PBS for 15 min, dried and incubated with 15 μl FITC-conjugated goat anti-rabbit Ig antiserum (Bethesda Research Laboratory) in a moist chamber (30 min, RT). The slides were washed again in PBS (15 min), dried and mounted in glycerol/PBS (9:1) containing 1 mg phenylenediamine/ml to prevent fading of the fluorochromes (15). A coverslip was sealed to the slide with paraffin wax with ceresin (BDH Chemicals, Poole, UK).

Microscopes

For evaluation of immunofluorescence and immunogold staining Zeiss and Leitz fluorescence microscopes were used. Both were equipped with phase contrast facilities and an adjustable aperture diaphragm in the epi-illumination system. The Zeiss microscope (Carl Zeiss, Oberkochen, FRG) was a Zeiss Universal II, equipped with a III RS Fluoreszenz-Auflicht condenser and a 50 W HBO mercury lamp (Osram, Berlin, FRG) and two 63x objectives (Carl Zeiss): neofluar 63/1.25 Oel Ph 3 and neofluar 63/1.25 Oel. Filter combination 19 (Carl Zeiss) was used for the evaluation of FITC and filter combination 14 (Carl Zeiss) for TRITC. The Leitz microscope (Ernst Leitz Wetzlar, Wetzlar, FRG) was a Leitz Orthoplan, equipped with a Leitz Ploemopak 2 illumination, a 100 W HBO mercury lamp (Osram), a Leitz Vario Orthomat camera and two 63x objectives (Leitz): PL APO 63/1.40 Oel Phaco 4 and 63/1.30 Oel Fluoreszenz. Filter combination I2 (Leitz) was used for evaluation of FITC and filter combination N2 (Leitz) for TRITC. For evaluation of colloidal gold, we used in both microscopes a polarization filter combination, consisting of a polarizer for the excitation light, a beamsplitter and an analyzer that extinguished reflected light by having the same polarization as the excitation light (11).

RESULTS

Microscopes

63x objectives with phase rings can be used for visualization of FITC and TRITC, but not for colloidal gold because of high background, probably due to reflection and depolarization of the polarized light by the phase rings. Visualization of the colloidal gold was made possible by the use of 63x objectives without phase rings and by closing the aperture diaphragm of the epi-illumination system. The best results were obtained by use of the Zeiss objective Antiflex-Neofluar 63/1.25 Oel which contains a rotatable quarter-wave plate. With the phase contrast dia-illumination system, the gold particles were not detectable, but by epi-illumination with polarized light the gold-yellow scattered light was clearly visible. For routine evaluation of triple staining the Zeiss microscope was used.

Triple immunologic staining

In the BM samples tested the percentage TdT positive cells ranged from 0.5 to 10%. The triple staining revealed that the majority (more than 70%) of the TdT positive cells was positive for HLA-DR, but negative for the T cell antigen CD7 (Figure 1). A minority (0.1-8%) of the TdT positive cells was positive for both HLA-DR and CD7. Detailed studies of these cell populations will be published elsewhere.

DISCUSSION

Colloidal gold particles are microscopically detectable by polarized light used in the same epi-illumination system as for fluorescence. Our results demonstrate that in triple immunologic staining, colloidal gold particles can be used as a third label in combination with the

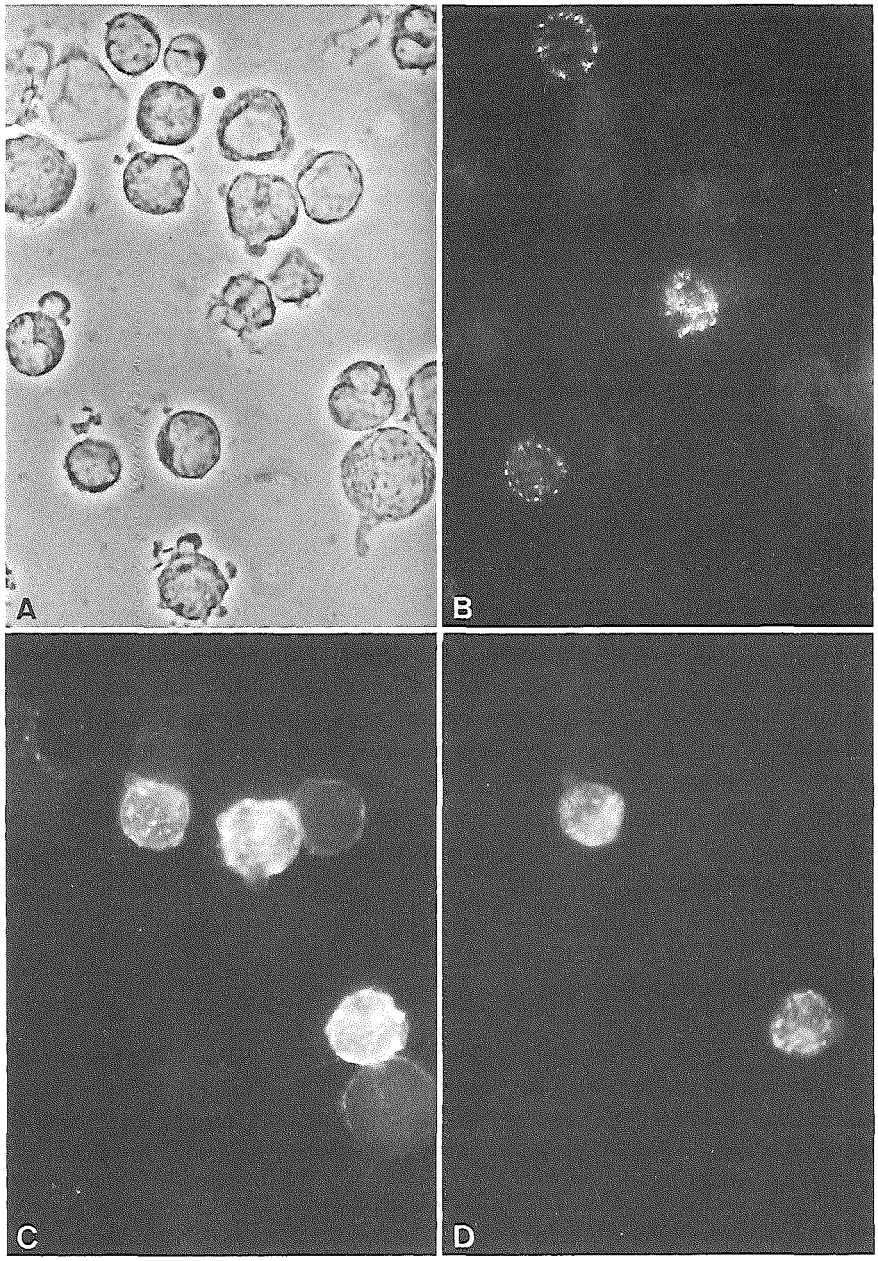


Figure 1. Triple immunologic staining of BM cells for CD7 (3A1), HLA-DR and TdT. **A**: phase contrast; **B**: CD7 (3A1) positive cells (colloidal gold labeled); **C**: HLA-DR positive cells (TRITC labeled); **D**: TdT positive cells (FITC labeled). The 2 TdT positive cells are also positive for HLA-DR, but not for the T cell marker CD7; they probably represent precursor B cells. Three cells are positive for CD7 and not for HLA-DR and TdT, probably representing normal T lymphocytes.

fluorochromes FITC and TRITC. The triple staining of BM cells for CD7, HLA-DR and TdT revealed that within the small TdT positive cell population subpopulations are present, e.g. a $CD7^-/HLA\text{-}DR^+/TdT^+$ subpopulation, and also a very small one of $CD7^+/HLA\text{-}DR^+/TdT^+$ phenotype. Thus triple immunologic staining can be a powerful tool in analyzing the heterogeneity of small cell populations in cell differentiation studies.

Since we used 2 mouse McAb and a rabbit antiserum in the triple staining, it was sufficient to use only 1 conjugated McAb. However, where combination of 3 mouse McAb is required, it has hitherto been necessary to use at least 2 conjugated McAb or McAb differing in Ig (sub)class in combination with differently conjugated anti-mouse Ig (sub)class antisera. A more elegant and less time-consuming solution is to use 3 differently conjugated McAb. Therefore it would be convenient if the McAb of the required specificities were not only available as FITC or TRITC conjugates, but also as colloidal gold conjugates.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof.Dr. R. Benner's support, and thank Mrs. E.G.M. Cristen, A.A. van der Linde-Preesman and I.L.M. Tettero for their excellent technical assistance, Dr. J. De Mey from Janssen Pharmaceutica and Dr. J.H.W. Leuvering from Organon International B.V. and Dr. M. De Waele for their advice, Mr. C.G. Enkelaar from Zeiss Nederland for his technical support, Mr. J. Fengler for his photographic assistance, and Mrs. C.J.M. Meijerink-Clerkx for typing this manuscript.

REFERENCES

1. Ploem JS. The use of a vertical illuminator with interchangeable dichroic mirrors for fluorescence microscopy with incident light. Z Wiss Mikr 1967;68:129-42.
2. Feller AC, Parwaresch MR. Simultaneous enzyme-immunocytochemical detection of antigens in monocellular specimens with monoclonal antibodies. J Immunol Methods 1983;63:273-9.
3. Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY. Immunoenzymatic staining of haematological samples with monoclonal antibodies. Br J Haematol 1983;55:395-410.
4. Schuit HRE, Hijmans W. Identification of mononuclear cells in human blood. II. Evaluation of morphological and immunologic aspects of native and formaldehyde-fixed cell populations. Clin Exp Immunol 1980;41:567-74.
5. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunologic monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res 1981;5:301-9.
6. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: Martinus Nijhoff Publishers, 1984:67-81.
7. Rothbarth Ph H, Tanke HJ, Mul NAJ, Ploem JS, Vliegenthart JFG, Ballieux RE. Immunofluorescence studies with 4-acetamido-4'-isothiocyanato stilbene-2,2'-disulphonic acid (SITS). J Immunol Methods 1978;19:101-9.
8. Titus JA, Haugland R, Sharrow SO, Segal DM. Texas red a hydrophilic, red-emitting fluorophore for use with fluorescein in dual parameter flow microfluorometric and fluorescence microscopic studies. J Immunol Methods 1982;50:193-204.
9. Oi VT, Glazer AN, Stryer L. Fluorescent phycobiliprotein conjugates for analyses of cells and molecules. J Cell Biol 1982;93:981-6.
10. Shapiro HM. Multistation multiparameter flow cytometry: a critical review and rationale. Cytometry 1983;3:227-43.
11. De Mey J. Colloidal gold probes in immunocytochemistry. In: Polak JM, Van Noorden S, eds. Immunocytochemistry, Practical application in pathology and biology. Bristol: Wright PSG, 1983:82-112.

12. Böyum A. Isolation of mononuclear cells and granulocytes from human blood. Scand J Clin Lab Invest 1968;21:77-89.
13. Haynes BF, Mann DL, Hemler ME, Schroer JA, Shelhamer JH, Eisenbarth GS, Strominger JL, Thomas CA, Mostowski HS, Fauci AS. Characterization of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans. Proc Natl Acad Sci USA 1980;77:2914-8.
14. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:293-9.
15. Johnson GD, De C Nogueira Araujo GM. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 1981;43:349-50.

CHAPTER 3

ANALYSIS OF IMMUNOGLOBULIN AND T CELL RECEPTOR GENES

CHAPTER 3.1

INTRODUCTION: ANALYSIS OF IMMUNOGLOBULIN AND T CELL RECEPTOR GENES

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

The surface membrane bound immunoglobulin (Ig) molecules and the T cell receptor (TcR) molecules represent the antigen-specific receptors of B and T lymphocytes, respectively (1-5). The diversity of these receptors is based on the fact that the variable parts of the Ig and TcR chains are encoded by combinations of gene segments, which differ in each lymphocyte (clone) (1-5). These different combinations of gene segments are obtained via processes of gene rearrangement, which start early during lymphoid differentiation (1-5).

The various types of lymphoid malignancies are generally regarded as clonal malignant counterparts of cells in the various stages of lymphoid differentiation (6-10). Because of their clonality and their availability in high cell numbers, these malignancies have been used to study the hierarchic order of Ig and TcR gene rearrangements during human lymphoid differentiation (11-24). Especially acute lymphoblastic leukemias (ALL) have been used for this purpose, because ALL represent the counterparts of normal precursor B cells and thymocytes (11-13,16-24). The use of T-ALL cell samples for studying human T cell differentiation will be discussed extensively in Chapter 4.

The detection of clonally rearranged Ig and/or TcR genes can be used for diagnostic purposes in patients with lymphoproliferative diseases, since clonality is equivalent to malignancy in the majority of cases. This is important when morphologic techniques and immunologic marker analysis cannot discriminate between a malignant and a reactive process (26-30).

In Chapter 3.2 we shall consider the structure of the antigen-specific receptors as well as the genetic basis of their diversity, i.e. the configuration of the Ig and TcR genes and their rearrangement during lymphoid differentiation. Several similarities and differences between Ig and TcR genes/molecules will be discussed. In Chapter 3.3 the methods for the analysis of Ig and TcR gene rearrangements as well as their pitfalls will be described. In addition, the diagnostic applications of such analyses will be indicated briefly. A more extensive description of the possibilities and limitations of Ig and TcR gene analysis for the diagnosis and management of lymphoproliferative diseases will be given in Chapter 6.

REFERENCES

1. Leder P. The genetics of antibody diversity. Sci Am 1982;246(5):102-15.
2. Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575-81.
3. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
4. Alt FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. Science 1987;238:1079-87.
5. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402 and corrigenda 1988;335:744.
6. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
7. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J. Immunol. 1981;126:1608-13.
8. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
9. Greaves MF. Differentiation-linked leukemogenesis in lymphocytes. Science 1986;234:697-704.
10. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.
11. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
12. Ha K, Hozumi N, Hrincu A, Gelfand EW. Lineage specific classification of leukaemia: results of the analysis of sixty cases of childhood leukaemia. Br J Haematol 1985;61:237-49.
13. Foa R, Migone N, Fierro MT, Basso G, Lusso P, Putti MC, Giubellino MC, Saitta M, Miniero R, Casorati G, Gavosto F. Genotypic characterization of common acute lymphoblastic leukemia may improve the phenotypic classification. Exp Hematol 1987;15:942-5.
14. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE, Whitcomb CC, Thomas CY. Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 1987;69:79-86.
15. Foroni L, Catovsky D, Luzzatto L. Immunoglobulin gene rearrangements in hairy cell leukemia and other chronic B cell lymphoproliferative disorders. Leukemia 1987;1:389-92.
16. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
17. Davey MP, Bongiovanni KF, Kaulfersch W, Quertermous T, Seidman JG, Hershfield MS, Kurtzberg J, Haynes BF, Davis MM, Waldmann TA. Immunoglobulin and T-cell receptor gene rearrangement and expression in human lymphoid leukemia cells at different stages of maturation. Proc Natl Acad Sci USA 1986;83:8759-63.
18. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
19. Goorha R, Bunin N, Mirro J, Murphy SB, Cross AH, Behm FG, Quertermous T, Seidman J, Kitchingman GR. Provocative pattern of rearrangements of the genes for the γ and β chains of the T-cell receptor in human leukemias. Proc Natl Acad Sci USA 1987;84:4547-51.
20. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ. T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
21. Foroni L, Foldi J, Matutes E, Catovsky D, O'Connor NJ, Baer R, Forster A, Rabbitts TH, Luzzatto L. α, β and γ T-cell receptor genes: rearrangements correlate with haematological phenotype in T cell leukaemias. Br J Haematol 1987;67:307-18.
22. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Comparison of T cell receptor α, β, and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J Clin Invest 1988;81:989-96.

23. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-82.
24. Van Dongen JJM, Wassenaar F, Wolvers-Tettero ILM, Borst J, Van den Elsen P. Expression of T cell receptor delta genes during human T cell differentiation: analysis of T cell acute lymphoblastic leukemias. Blood 1989;74:334-42.
25. Korsmeyer SJ. Antigen receptor genes as molecular markers of lymphoid neoplasms. J Clin Invest 1987;79:1291-5.
26. O'Connor NTJ, Gatter KC, Wainscoat JS, Crick J, Jones DB, Delsol G, Ralfkiaer E, De Wolf-Peeters C, Angus B, Mason DY. Practical value of genotypic analysis for diagnosing lymphoproliferative disorders. J Clin Pathol 1987;40:147-50.
27. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.
28. Cossman J, Uppenkamp M, Sundeen J, Coupland R, Raffeld M. Molecular genetics and the diagnosis of lymphoma. Arch Pathol Lab Med 1988;112:117-27.
29. Sklar J, Weiss LM. Applications of antigen receptor gene rearrangements to the diagnosis and characterization of lymphoid neoplasms. Annu Rev Med 1988;39:315-34.
30. Knowles DM. Immunophenotypic and antigen receptor gene rearrangement analysis in T cell neoplasia. Am J Pathol 1989;134:761-85.

CHAPTER 3.2

ANTIGEN-SPECIFIC RECEPTORS OF LYMPHOCYTES*

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

The ability of the immune system to recognize specifically multiple antigens or antigenic epitopes is based on the enormous diversity (at least 10^7 to 10^9) of antigen-specific receptors, namely surface membrane bound immunoglobulin (SmIg) on B lymphocytes and T cell receptor (TcR) molecules on T lymphocytes (1-4). When the Ig or TcR molecules of a particular B or T lymphocyte recognize the antigen against which the receptor is directed, lymphocyte activation and proliferation will follow. All cells derived from one lymphocyte have identical antigen-specific receptors and together they form a clone. The B lymphocytes derived from this process can mature to plasma cells which secrete antigen-specific antibodies (Ig molecules), while activated T lymphocytes can exhibit their specific regulatory and cytotoxic functions (2-4).

Ig molecules are composed of two Ig heavy (IgH) chains and two Ig light (IgL) chains, held together by disulfide bonds (Figure 1)(5). The IgL chains of a B lymphocyte or B cell clone are of the same type: Igκ or Igλ. The TcR is composed of two chains, which are generally also linked by a disulfide bond (6,7). On the cell surface the TcR is closely associated with CD3 molecules and together they form the TcR-CD3 complex (Figure 1)(6-9). The CD3 structure consists of at least five protein chains and is probably involved in signal transduction from the TcR into the cytoplasm (10-12). Each of the Ig chains and TcR chains consists of a constant (C) region as well as a variable (V) region which is involved in antigen recognition (Figure 1).

If the entire repertoire of the human Ig and TcR chains would be encoded by separate genes, they would occupy a large part of the human genome. Instead, a limited number of gene segments is able to code for the receptor diversity due to the fact that the V regions of the Ig and TcR chains are encoded by combinations of gene segments, which are different for each lymphocyte or lymphocyte clone (13-15). The joining of these gene segments is obtained via a process of gene rearrangement (13-15). In 1987 the Nobel prize was awarded to Dr. Susumu Tonegawa for his contribution to the unraveling of the genetic mechanisms, which allow the encoding of so many different antigen-specific receptors by a relatively small number of gene segments (14,16,17).

This manuscript will be published as part of a review in Clin Chim Acta.

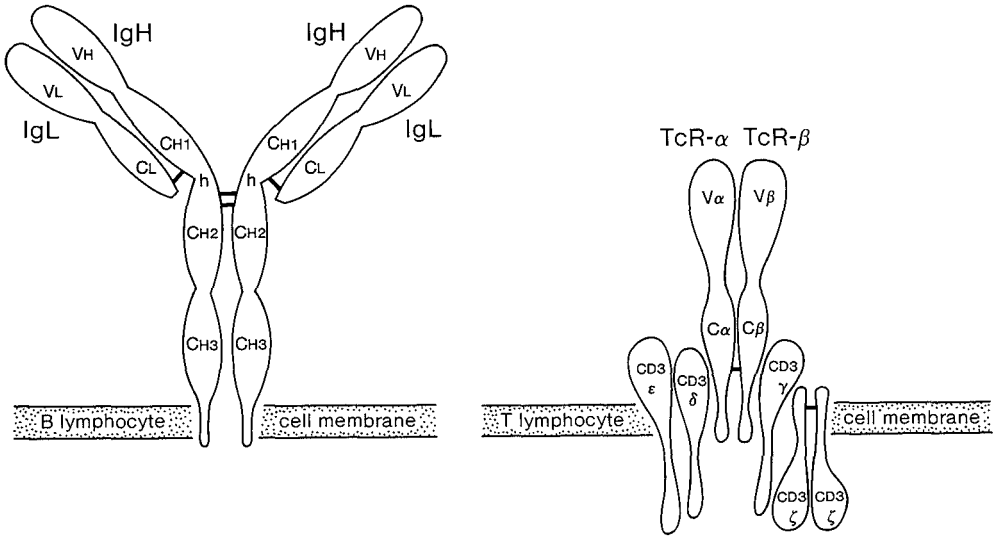


Figure 1. Schematic diagram of an Ig molecule and a TcR-CD3 complex on the cell membrane of a B lymphocyte and a T lymphocyte, respectively. VH: variable domain of IgH chains; VL: variable domain of IgL chain; CH constant domain of IgH chain; CL: constant domain of IgL chain; h: hinge region; Vα: variable domain of TcR-α chain; Vβ: variable domain of Vβ chain; Cα: constant domain of TcR-α chain; Cβ: constant domain of TcR-β domain; CD3-γ, δ and ε: protein chains of the CD3 molecule.

IMMUNOGLOBULIN GENES AND T CELL RECEPTOR GENES

Immunoglobulin molecules and their encoding genes

Five different classes of human Ig molecules are known (IgM, IgD, IgG, IgA and IgE). Within the classes of IgG and IgA molecules several subclasses can be recognized: IgG1, IgG2, IgG3 and IgG4 as well as IgA1 and IgA2. The class or subclass (isotype) of an Ig molecule is determined by the constant region of the IgH chain expressed (μ, δ, γ1, γ2, γ3, γ4, α1, α2, or ε), independent of the type of IgL chain (5). The constant region of the IgH chains are responsible for effector functions such as transmembrane signalling, complement activation and binding to Fc receptors (5). A B lymphocyte will irreversibly express only one type of IgL chain (κ or λ), whereas different IgH chains can be expressed: i.e. different IgH constant regions, linked to the same variable region (18,19). The majority of B lymphocytes in peripheral blood express IgM and IgD on the cell membrane, whereas a minority express IgG or IgA. In lymph nodes and other lymphoid tissues higher frequencies of IgG and IgA bearing lymphocytes are present.

The IgH and IgL chains are composed of several domains, which consist of roughly 110 amino acids (Figure 1)(5). The variable regions of Ig chains consist of one domain. Also the IgL constant region consists of a single domain (CL), but most IgH constant regions are composed of three domains (CH1, CH2 and CH3) and a hinge region (h), which is located between CH1 and CH2 (Figure 1)(5). The hinge region creates a high flexibility within the Ig molecule, which allows bivalent recognition of variably spaced antigenic determinants. The

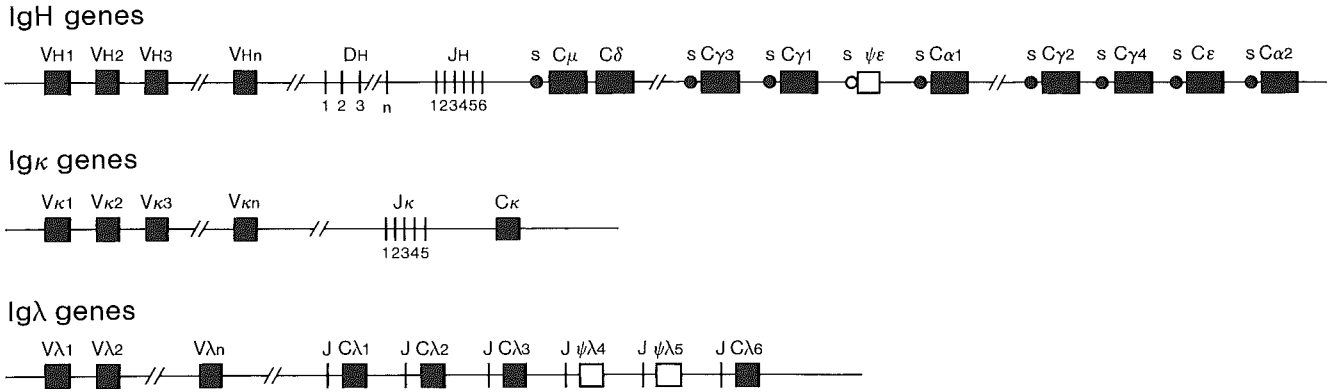


Figure 2. Schematic diagram of the human Ig genes. The IgH genes consist of many V genes, at least ten D genes, about six J genes, and C genes for the various IgH classes and subclasses (20-24). Most C genes are preceded by a switch gene (s), which plays a role in IgH (sub)class switch (19,38-41). The Igκ gene complex consists of a series of V genes, about five J genes and one C gene (42,43), while the Igλ gene complex consists of V genes and six C genes, all of which are preceded by a J gene (44-46). Pseudo genes (ψ) are indicated with open symbols.

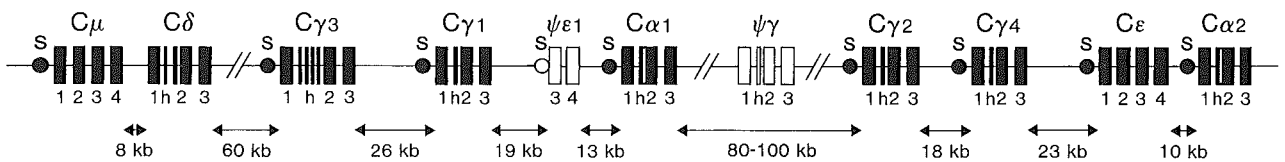


Figure 3. Schematic diagram of the organization of the human IgH constant genes on chromosome 14 based on data from the literature (c.f. references 24-35). The various exons which code for the C domains and the hinge regions (h) as well as the switch sequences (s) are indicated. In case of the Cα genes, the hinge region sequences are included in the second C exon (32). The distances between the C region genes are given (see references 24,25,28-30,50-52), but the diagram is not on scale. The complete locus of IgH-C genes is located within a region of 300-320 kb (51,52). In addition to the functional Cγ genes one pseudo γ gene ($\psi\gamma$) without preceding switch sequences is located between Cα1 and Cγ2 (30,50). Also two pseudo ε genes ($\psi\epsilon1$ and $\psi\epsilon2$) are known (33-37). The decapitated $\psi\epsilon1$ gene is located between Cγ1 and Cα1 on chromosome 14 (24,35), while the $\psi\epsilon2$ gene (consisting of one large exon without intervening sequences) is located outside the IgH gene locus, i.e. on chromosome 9 (36).

flexibility is very high in IgG3 molecules due to an extended hinge region (5). In case of IgM and IgE molecules the hinge region is replaced by a constant domain; the μ and ϵ chains therefore have four CH domains (5).

Like many genes in eukaryotic cells, the Ig genes consist of encoding gene segments (exons), separated by intervening non-coding gene segments (introns). Each domain of an Ig chain is encoded by a separate exon. The variable region domain of the Ig chains is encoded by an exon, which consists of a combination of a V (variable), a D (diversity) and a J (joining) gene segment in case of the IgH chain or a combination of a V and a J gene segment in case of the IgL chain (13,14). During B cell differentiation these V, (D,) and J gene segments are joined through a process of gene rearrangement (13,14). The constant domains of the Ig chains are encoded by a C (constant) gene.

The IgH gene locus contains a large number of V gene segments (20), at least ten D genes (21-23) and about six functional J genes (21). For each IgH isotype a separate CH gene exists (24). Each CH gene consists of several exons: one exon for each CH domain and one or more exons for most hinge regions (25-37). The IgH-C genes, except for the Cδ gene, are preceded by a switch gene (S), which is necessary for isotype switching (see below)(18,19,25,38-41). The Igκ gene locus consists of a series of Vκ genes, five Jκ genes and only one Cκ gene (42,43). Finally, the Igλ gene locus contains several Vλ genes and six virtually identical Cλ genes, which each are preceded by one Jλ gene segment (44,45). Two of these Cλ genes are non-functional genes (pseudogenes)(46). In about 25% of individuals even more than six Cλ genes are present on one or both alleles (47). Recently, new Igλ genes have been identified, which are located outside the "classical" Igλ gene locus (48,49).

The germline (non-rearranged) configuration of the IgH, Igκ and Igλ genes is illustrated in Figure 2, while the separate exons for the constant domains and the hinge regions of the IgH chains are presented in Figure 3 (25-37). Figures 2 and 3 illustrate that during evolution a duplication of IgH-C genes has probably occurred, involving the Cγ, Cϵ and Cα genes (24,30,32, 35,50). The complete cluster of functional CH genes is located within 300-320 kilobases (kb) (51,52). Within the Igλ gene locus a multiplication of Jλ-Cλ gene segments has occurred during evolution (44,47).

T cell receptor molecules and their encoding genes

Two different types of TcR have been recognized: the classical TcR, which consists of a TcR-α chain and a TcR-β chain (TcR-$\alpha\beta$)(3,4,6) and the "alternative" TcR, which is composed of a TcR-γ and a TcR-δ chain (TcR-$\gamma\delta$)(53-55). The majority of mature T lymphocytes (85 to 98%) in peripheral blood and most lymphoid tissues express TcR-$\alpha\beta$, while a minority (2 to 15%) expresses TcR-$\gamma\delta$ (56,57). Interestingly, intraepithelial lymphocytes in the murine intestine mainly represent TcR-$\gamma\delta^+$ T lymphocytes (58,59). This epithelial tropism of TcR-$\gamma\delta^+$ cells has not been found in man (60,61).

In contrast to the various isotypes of IgH and IgL chains, the four different TcR chains do not randomly pair with each other. For instance, no TcR-$\alpha\gamma$ or TcR-$\beta\gamma$ have been identified so far. Even cell lines which produce complete TcR-β, TcR-γ and TcR-δ chains only express TcR-$\gamma\delta$ (62,63) and transfection of functional TcR-α and/or TcR-β gene sequences into TcR-$\gamma\delta^+$ cells did not result in the expression of aberrant TcR, although some of the transfectants expressed

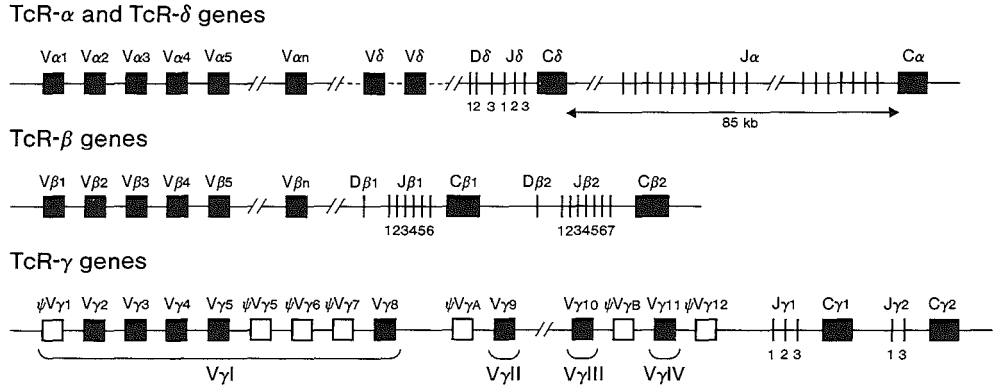


Figure 4. Schematic diagram of the human TcR genes. The TcR-α genes consist of many V genes, a remarkably long stretch of J genes and one C gene (66-72). The TcR-β gene complex consists of a series of V genes and two C genes, both of which are preceded by one D gene and six or seven J genes (73-76). The TcR-γ genes consist of a restricted number of V genes (eight functional Vγ genes and seven pseudo genes) and two C genes, each of which are preceded by two or three J genes (77-82). Interestingly, the TcR-δ genes are located between the Vα and Jα genes and probably consist of a few V genes, three D genes, three J genes and one C gene (83-87).

both TcR-αβ and TcR-γδ (63,64). Nevertheless the human T cell line DND41 was proven to express a βδ receptor (65), which has not been found on normal T lymphocytes so far.

The TcR chains are composed of two domains: a variable domain and a constant domain (Figure 1). Analogous to the Ig chains, the variable domains of the TcR chains are encoded by a combination of a V and a J gene in case of the TcR-α and the TcR-γ chain or by a combination of V, D and J genes in case of the TcR-β and the TcR-δ chain (66-87). The constant domains of the TcR-chains are encoded by C genes. One C gene codes for the constant domain of the TcR-α chain and one for the TcR-δ chain, while two C genes are available for the constant domains of the TcR-β and TcR-γ chains (66-87).

The germline configuration of the four TcR genes is presented in Figure 4. The TcR-α genes consist of many Vα genes, a remarkably long stretch of Jα genes (about 85 kb) and one Cα gene (66-72). Interestingly, the TcR-δ gene locus is located within the TcR-α gene complex between the Vα genes and the long stretch of Jα genes (69-72,84). The TcR-δ genes consist of a few Vδ genes, three Dδ genes, three Jδ genes and one Cδ gene (69-72,83-87). Figure 4 illustrates that both Cβ gene segments are preceded by a Dβ gene and six or seven Jβ genes (73-76) and that both Cγ genes are preceded by two or three Jγ genes (77,78,80).

Analysis of the nucleotide sequence of the two Cβ genes revealed that they are highly homologous (76,88). High levels of homology were also found between the two Jγ-Cγ gene segments (77,80,89). This suggests that during evolution a duplication has occurred within the TcR-β gene locus as well as within the TcR-γ gene locus. Nevertheless, several differences exist between the duplicated gene segments. This is clearly illustrated by a difference between the two Cγ gene segments (89-91).

A TcR-γδ, in which the TcR-γ chain is derived from Cγ1 genes has an interchain disulfide bond, comparable to the disulfide bond between the protein chains of TcR-αβ (Figure 1)(92-94).

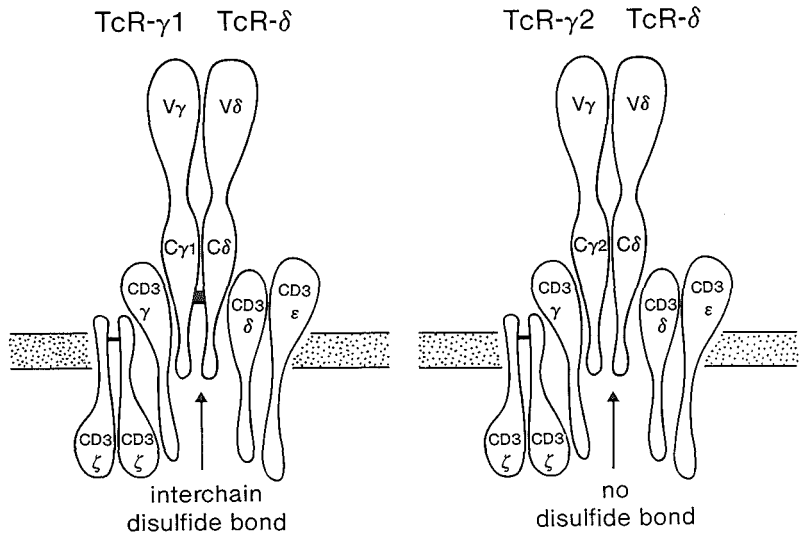


Figure 5. Schematic diagram of the two main types of TcR-γδ receptors. The protein chains of the TcR-γ1δ receptor are disulfide linked, while this interchain disulfide bond is lacking in TcR-γ2δ receptors (92-94).

However, if the TcR-γ chain is derived from Cγ2 genes, the interchain disulfide bond is lacking (Figure 5)(92-94). Disulfide bonds are formed between cysteine residues. The cysteine residue which is responsible for the interchain disulfide bond is coded by a TGT codon in the second exon of the Cγ1 gene (89,90,92). Although the second exon in the Cγ2 gene is duplicated (or even triplicated in one third of individuals), no cysteine codon is present (89-92). The far majority of the TcR-$\gamma\delta^+$ T lymphocytes in peripheral blood appear to express the disulfide linked receptor (54,55,95). It is not clear whether Cγ1 and Cγ2 encoded TcR-γδ define functionally distinct lineages. It has been suggested that TcR-γ1δ T lymphocytes exhibit a higher degree of cytotoxicity than the TcR-γ2δ T lymphocytes (96). Also differences in morphology, cytoskeletal organization and growth characteristics have been found (97).

Chromosomal localization and orientation of the Ig and TcR genes

Human-rodent somatic cell hybrids, which segregate human chromosomes, have proven to be useful for gene localization studies (98,99). Correlations between human protein expression by hybrid cells and the presence of specific human chromosomes or fragments of these chromosomes allow the assignment of the encoding genes to the correct chromosome or chromosome fragment (98,99). If DNA sequences of the involved gene are available, DNA extraction from hybrid cells with a known human chromosome content and its subsequent analysis for the presence of the encoding gene can be used for gene mapping (100). The availability of the appropriate DNA sequences also allows the application of a completely different approach in which somatic cell hybrids are not needed: *in situ* hybridization of labeled DNA sequences

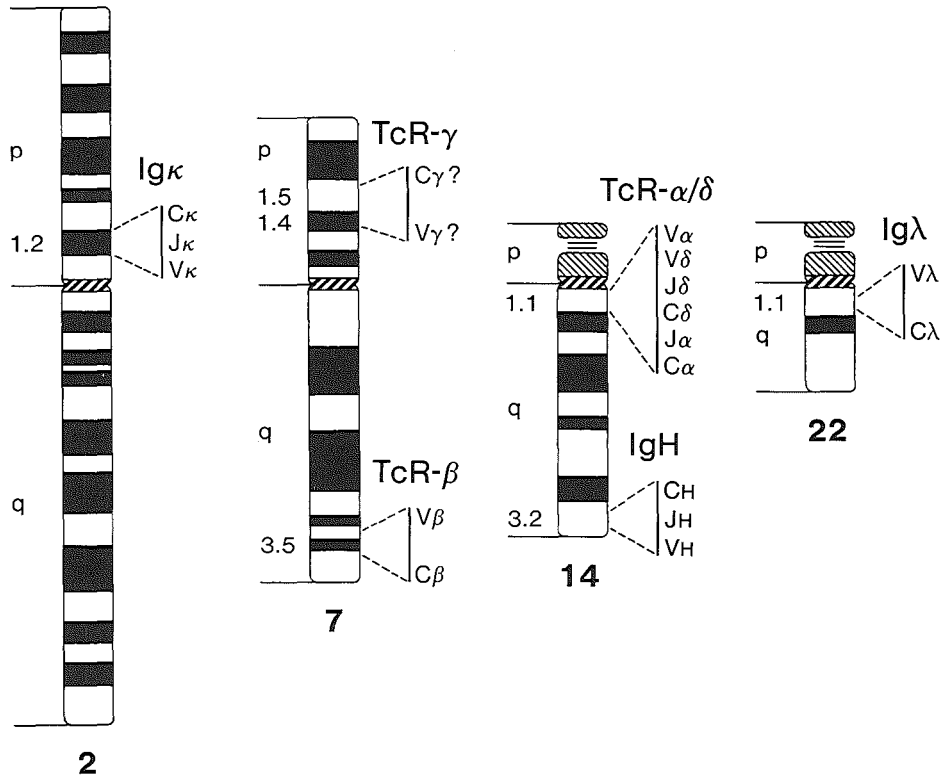


Figure 6. Location and orientation of the three Ig genes and the four TcR genes on chromosomes 2, 7, 14 and 22 (103-127). The orientation of the Igκ, Igλ, TcR-β and TcR-α/δ gene complexes is such that the V regions are located at the centromeric side and the C regions at the telomeric side (120-127). In case of the IgH genes, the V segments are telomeric and the C genes centromeric (119). The orientation of the TcR-γ genes on chromosome 7 is not yet known.

to fixed human metaphase chromosomes (101,102). The latter technique enables a more precise regional localization of genes (101,102).

All three techniques have been used for mapping of the Ig and TcR genes. The IgH genes were assigned to chromosome 14 (band q32)(103,104), the Igκ genes to chromosome 2 (band p12)(105,106) and the Igλ genes to chromosome 22 (band q11)(105,107,108). The TcR-α genes as well as the TcR-δ genes appear to be located on chromosome 14 (band q11)(84,109-113), the TcR-β genes on chromosome 7 (band q35)(113-117) and the TcR-γ genes also on chromosome 7 (bands p14-15)(113,118).

Lymphoid malignancies with chromosomal aberrations involving the Ig and TcR gene loci, can be used for both fine mapping of the genes as well as for determining the orientation of the genes on the chromosomes. In this way the orientation of all Ig and TcR genes, except for the TcR-γ gene, has been established (Figure 6)(72,84,119-127).

Breakpoints in chromosome bands containing the Ig and TcR genes are not only frequently found in translocations in lymphoid malignancies (128-131), but also in the recurrent chromosomal

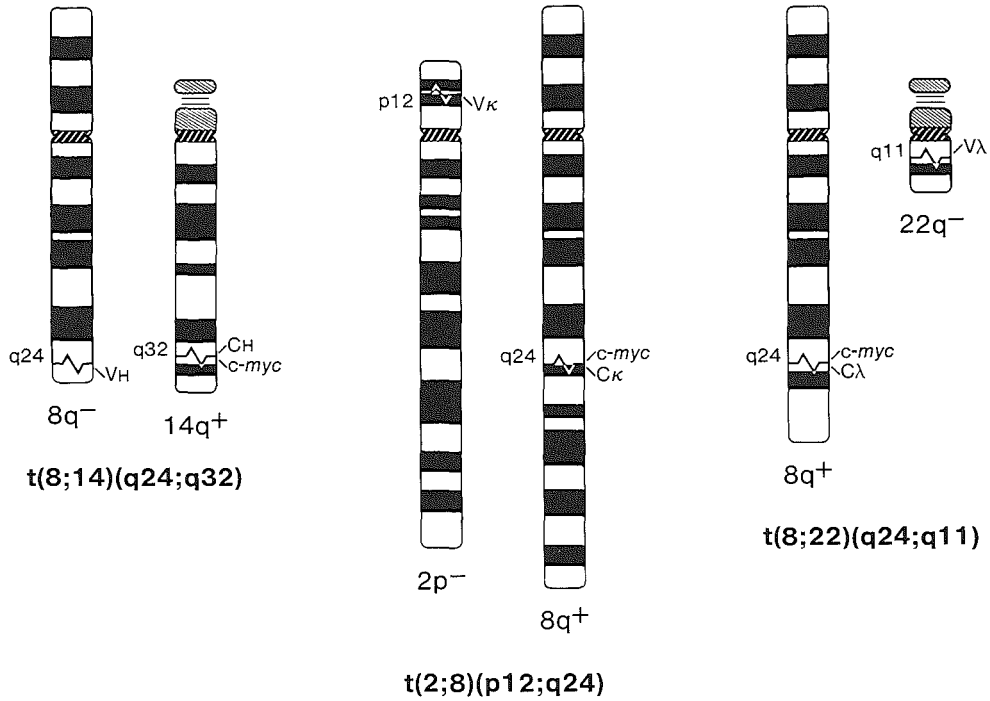


Figure 7. Schematic diagram of the three Burkitt translocations. In t(8;14)(q24;q32) the c-*myc* oncogene is translocated from chromosome 8 to the IgH genes in band 14q32 (147,148), while in the two variant Burkitt translocations t(2;8)(p12;q24) and t(8;22)(q24;q11) the telomeric parts of the Igκ and Igλ genes are translocated to the c-*myc* gene in band 8q24 (147,148).

aberrations in the lymphoid cells of patients with ataxia telangiectasia (132-137); such chromosomal aberrations are occasionally found in lymphocytes from normal individuals (132,134,138-143). Ataxia telangiectasia (AT) is a rare complex multisystemic disease (including immunological defects) and is characterized by chromosomal instability, a DNA repair/processing defect and a high tendency to develop leukemia or non-Hodgkin lymphoma (NHL)(144). These data indicate that the Ig and TcR genes are highly vulnerable to breakage and that such breaking points may play a role in the oncogenic events which lead to the development of a lymphoid malignancy. This is illustrated by the so-called Burkitt translocations t(8;14)(q24;q32), t(2;8)-(p12;q24) and t(8;22)(q24;q11) in which one of the Ig genes and the c-*myc* oncogene (located in band 8q24) are involved (119-121,145-148)(Figure 7).

DIVERSITY OF THE ANTIGEN-SPECIFIC RECEPTORS

As mentioned above, the enormous diversity of antigen-specific receptors of lymphocytes is based on the rearrangement of gene segments which code for the variable domains of the Ig and TcR molecules (13-15). The extent of the whole repertoire of Ig and TcR molecules is

mediated by : 1, the diversity of the germline genome (i.e. the number of V, D and J segments); 2, the combinatorial diversity (i.e. the number of possible V-(D-)J combinations); and **3**, the junctional diversity (i.e. diversity due to the imprecise joining of the V, D and J segments)(13-15,149). After the standard V-(D-)J rearrangements secondary rearrangements may occur, which represent replacements of previously rearranged gene segments (149). All the genomic rearrangement events together generate the primary repertoire of the antigen receptors, i.e. the variable domains of the Ig and TcR chains (13-15,149).

Within the variable domain of each Ig chain the antigen binding site is composed of three hypervariable regions, the so-called *complementarity determining regions (CDR)*(150-154). Two of these CDR are encoded by germline sequences of the V gene, while the third CDR (CDR3) is encoded by the junctional region of the V-(D-)J segments (150-154). Therefore this junctional region directly influences the antigen binding of the Ig molecules. In analogy to the Ig molecules also the variable domains of TcR chains contain CDR-equivalent regions in their variable domains (155-157). Also here the CDR3-equivalent region probably corresponds to the junctional region (156,157).

The primary antibody repertoire of Ig molecules can be enhanced via antigen-induced somatic mutations in the V gene segments of Ig genes (158,159). So far, somatic mutations in V genes have not been reported for TcR genes. The secondary antigen-dependent mutation processes in Ig-V genes will be discussed in one of the next sections.

Several aspects of the gene rearrangement processes as well as the various mechanisms for the development of the primary (antigen-independent) repertoire of Ig and TcR molecules will be discussed below.

Gene rearrangement: V-(D-)J joining

During B and T cell differentiation the germline V, (D,) and J segments rearrange and each lymphocyte thereby obtains its own specific combination of V-(D-)J segments (13-15). An example of an IgH gene rearrangement is illustrated in Figure 8: first one of the J gene segments is joined to one of the D gene segments and subsequently a V to D-J joining occurs, thereby deleting all intervening sequences. After the process of rearrangement the gene is transcribed into a precursor mRNA, which becomes a mature mRNA after splicing and eliminating all non-coding intervening sequences (Figure 8)(13). Virtual identical rearrangement and transcription processes occur in the other Ig and TcR genes (13-15).

The gene rearrangement processes are mediated by an, as yet, unidentified recombinase enzyme system, which recognizes specific joining sequences. These joining sequences consist of a palindromic heptamer and nonamer, separated by spacer regions of 12 or 23 base pairs (14,160,161). A complete joining sequence, starting with the heptamer, borders the 3′ side of each V and D segment and the 5′ side of each D and J segment (13,14,160,161). A gene rearrangement first involves a back-to-back fusion of the heptamer-nonamer sequences. This is followed by deletion of these joining sequences as well as the sequences between them in the form of a circle and by subsequent joining of the two gene segments (Figure 9)(13,14). Indeed circular TcR gene excision products could be isolated from thymocytes. Gene segments of all four TcR genes have been identified in such circular DNA products (162-165).

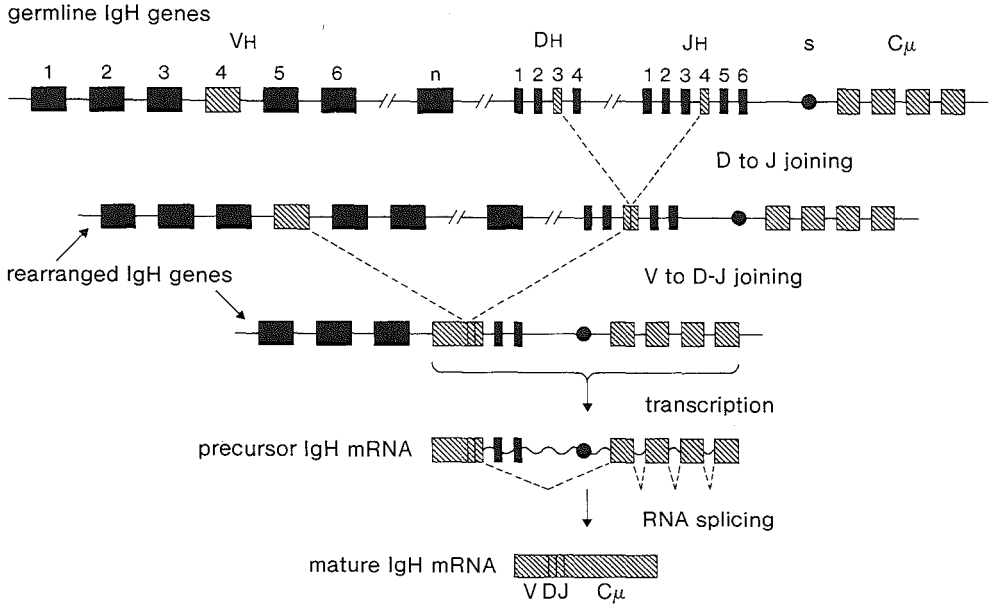


Figure 8. Schematic diagram of rearrangement and transcription of IgH genes. First D to J joining occurs, followed by V to D-J joining. The rearranged genes can be transcribed into a precursor IgH mRNA, which becomes a mature IgH mRNA after splicing all non-coding intervening sequences (13,149).

The heptamer-nonamer recognition sequences of the Ig and TcR genes are well conserved during evolution (166,167). This is illustrated by the high homology of the heptamer-nonamer sequences, which flank the V, (D,) and J segments of the Ig and TcR genes (166,167). Furthermore, several experiments have demonstrated that the artificial introduction of Ig or TcR gene sequences (containing heptamer-nonamer sequences) into immature B cells results in site-specific joining events of the introduced Ig or TcR genes (167-170). These data suggest that B and T cells use a common recombinase for their receptor gene rearrangements (167-170).

Junction of two gene segments can not only be obtained via deletion of the intervening sequences (Figure 9), but also via inversion without deletion of gene segments (149,166). The latter process of inversion joining has occasionally been found in IgH genes (171,172), TcR-β genes (173,174) and TcR-δ genes (87,175) and seems to occur more frequently in murine Igκ genes (176,177).

Secondary gene rearrangements

Ig and TcR gene rearrangements are complex processes in which the joinings of the gene segments are imprecise (14,149,171). Because of the triplet reading frame of DNA sequences, approximately two out of three joinings will be aberrant, i.e. a mRNA is produced which cannot be transcribed into a complete protein. The high frequency of aberrant rearrangements explains why most B cells have rearranged both IgH gene alleles and why most T cells have biallelic

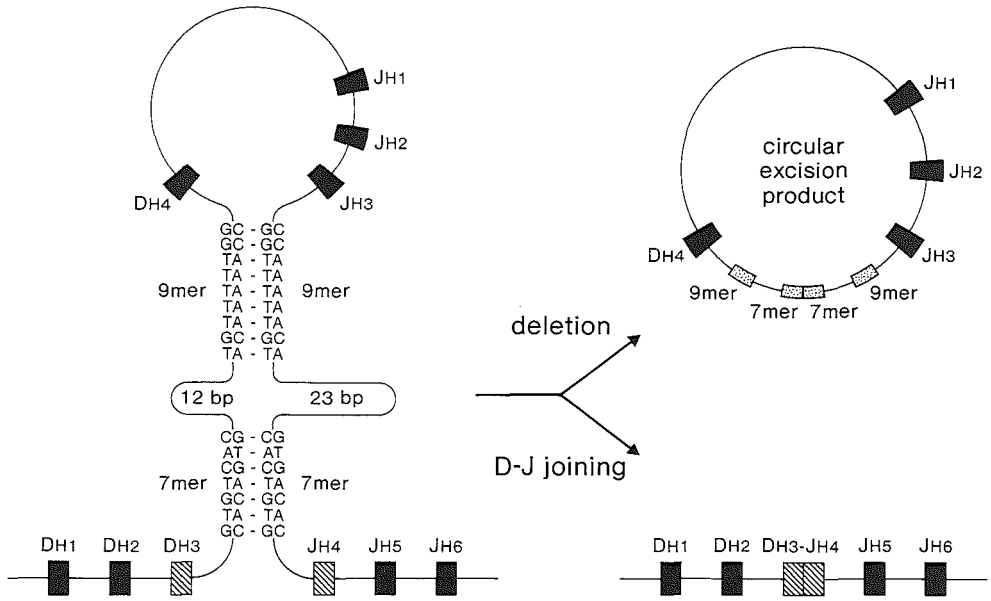


Figure 9. Schematic diagram of the function of joining sequences during gene rearrangement. The 3′ DH3 and 5′ JH4 heptamer-nonamer sequences fuse back to back. This is followed by a DH3 - JH4 joining and the deletion of a circular excision product (162-165). The given heptamer-nonamer sequences are not the exact 3′ DH3 and 5′ JH4 joining sequences, but represent the consensus heptamer-nonamer sequences which are well conserved in Ig as well as TcR genes (166,167).

rearrangements of their TcR-β and TcR-γ genes (178-183).

In addition to the biallelic rearrangements, secondary gene rearrangements appear to occur in order to rescue precursor B and T cells with two non-productive Ig or TcR genes (184-186). In B cells two types of secondary rearrangements have been described: *D-JH replacements* and *V to VDJH rearrangements* (184-186). In T cells replacements of preexisting V-J rearrangements have been reported to occur in TcR-α genes (164,187-189) and V gene replacements may probably occur in TcR-β genes (190,191).

The secondary D-JH rearrangements in B cells replace the whole preexisting out-frame DJH complex by joining an upstream DH gene to a downstream JH gene. Due to the multiple element organization of the DH and JH gene clusters, *D-J replacements* may occur repeatedly on the same chromosome as long as it carries unrearranged DH and JH elements (184). Similar replacement processes may also occur in Igκ and Igλ genes, in which V-J complexes can potentially be replaced (184). Indeed multiple rearrangements on the same allele have been observed in the murine Igκ locus (176,177).

The other type of secondary rearrangement in B cells is the *V to V-D-JH rearrangement* in which the 'old' VH gene is replaced by a new upstream VH gene (185,186). The joining in this rearrangement is mediated by a heptamer joining sequence found in the 3′ region of most VH genes (Figure 10)(185,186). Interestingly, VH gene replacements do not only occur in B cells with two aberrant V-D-J rearrangements (185), but may also occur in productive VDJH alleles,

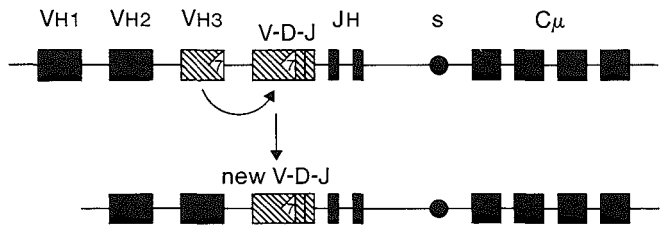


Figure 10. Schematic diagram of V to V-D-JH rearrangement. In this example the VH4 gene segment is replaced by the VH3 gene segment via a rearrangement process in which heptamer (indicated as: 7) joining sequences are involved (185,186).

resulting in a new productive rearrangement (186).

Internal V heptamer joining sequences, which allow the V to V-D-JH rearrangements in IgH genes, are not present in the V segments of IgL and TcR loci, except for the TcR-Vγ genes (185). Nevertheless changes in antigen specificity of cytotoxic T lymphocytes were attributed to V gene replacements in functionally rearranged TcR-β genes (190,191).

In recent studies it was demonstrated that the protein chemical differences between TcR-αβ molecules of subclones and their parental T cell line were due to Vα-Jα replacements (187,189). In these secondary rearrangements preexisting productive and non-productive Vα-Jα complexes were replaced by joining an upstream Vα segment to a downstream Jα gene. The occurrence of Vα-Jα replacements was confirmed by the presence of rearranged Vα-Jα gene segments in circular excision products, isolated from thymocytes (164,188). It was suggested that these Vα-Jα replacements may play a role in the thymic selection of the T cell repertoire (187).

Germline diversity and combinatorial diversity

Based on the above described gene rearrangements many different combinations of V-D-J complexes can be obtained, depending on the number of V, D and J gene segments within the gene locus.

The IgH gene locus probably contains 100 to 200 VH genes, which are grouped into six families based on homology (20,192-195). In addition, at least ten DH genes and about six functional JH genes exist (21,22). Thus, assuming a random paring of the gene segments during the rearrangement processes, at least 6000 different V-D-JH complexes can be made (Table 1). For the Igκ genes (40-80 Vκ genes and about 5 Jκ genes) about 400 combinations can be made (42,43,196-198), while for the Igλ genes (over 40 Vλ genes and at least 4 functional Jλ genes) at least 160 combinations are available (45,46,198-200). Since an Ig molecule consists of IgH and IgL chains, the pairing of an IgH chain with an Igκ or an Igλ chain will result in at least 2-4 million combinatorial possibilities (Table 1).

Also for the TcR-αβ and TcR-γδ receptors calculations for combinatorial diversity can be made. The TcR-α genes do not have D segments, but contain more than 50 Vα genes and a remarkably long stretch of about 55 Jα genes (67,70,201-204). Together with the various V-D-Jβ combinations

TABLE 1. Estimation of the potential primary repertoire of human Ig and TcR molecules.

	Ig molecules			TcR-αβ molecules		TcR-γδ molecules	
	IgH	Igκ	Igλ	TcR-α	TcR-β	TcR-γ	TcR-δ
Germline diversity; number of genes							
- V genes	100-200	40-80	>40	>50	>70	8[b]	≥6
(families)[a]	(6)	(4)	(7)	(19)	(20)	(4)	(6)
- D genes	>10	–	–	–	2	–	3[c]
- J genes	6	5	>4	about 55	13	5	3
Combinatorial diversity							
- per chain	6000-12,000	200-400	>160	>2750	>1820	40	>75
- per complete molecule		>2-4 x 10^6		>5x10^6		>3000	
Junctional diversity							
- number of joining sites	2	1	1	1	2	1	1-4[c]
- N region insertion	++	–	–	+	++	++	++++
- estimated degree of increase in diversity	x10^4			x10^2	x10^4	x10^3	x10^9
Estimation of total primary receptor repertoire		>10^{10}		>10^{12}		>10^{15}	

a. The various V genes are grouped into families based on homology (references 79,81,82,175,192-208). The number of V gene families is given in parentheses.
b. In addition to the eight functional Vγ genes seven pseudogenes have been described (references 79,81,82,175).
c. The variable domain of the TcR-δ chain may not only be encoded by V-D-J complexes, but also by a V-J complex or even by V-D-D-J or V-D-D-D-J complexes (references 70,87,211,213).

at least five million different TcR-αβ receptors can be produced (Table 1)(203-206).

The combinatorial diversity of the TcR-γδ receptors appears to be limited, because only eight functional Vγ genes and five Jγ genes are available in addition to at least six Vδ genes, three Dδ genes and three Jδ genes (Table 1)(79-82,86,97,175,207,208). Since the TcR-δ genes are located between the Vα and Jα gene segments, the TcR-δ and TcR-α chains can potentially share the very same pool of V genes (69-71). The usage of a V gene from the Vα6 gene family by a TcR-δ chain has been demonstrated in a T cell clone obtained from a patient with an immunodeficiency (201,209). Similarly, in the mouse some V segments rearranged to TcR-δ gene segments appeared to be virtually identical to V segments used in TcR-α gene rearrangements, while another Vδ gene showed more than 90% homology with a Vα gene segment (164,210,211). This raises the possibility that TcR-δ chains can draw their V genes from a much larger V gene pool. Another curious phenomenon concerning the TcR-δ chain is that the variable part of the TcR-δ chain may not only be encoded by V-D-Jδ complexes (83,212), but also by V-Jδ complexes, V-D-D-Jδ complexes, and even V-D-D-D-Jδ complexes (70,87,211,213). Both phenomena increase the combinatorial diversity of the TcR-γδ receptors to at least 3 x 10^3 (Table 1).

The above described calculations of the combinatorial diversity are based on the assumption that the various V, D and J gene segments are used at random. However, this seems not always to be the case (149). For instance, fetal B cells seem to use a restricted set of VH segments, related to JH proximity (149,194,214). Also preferential use of DH and JH

segments has been described (149,194,215). In addition, preferential use of the Jβ2.5 gene segment in certain TcR-$\alpha\beta^+$ cells has been described (216) and TcR-$\gamma\delta^+$ lymphocytes in blood appear to use a restricted number of the available Vγ and Vδ genes (217-220). On the other hand, it may well be that the whole possible combinatorial repertoire is present, but that certain receptor specificities dominate due to clonal selection/expansion.

Junctional diversity (N region diversity)

As indicated above the joining of the V, (D,) and J segments is imprecise (14,171). This imprecision is manifested by loss of original nucleotides as well as by *de novo* insertion of nucleotides between the joined gene segments (14,171,221). This insertion of nucleotides (N region insertion) at the junction sites is most probably mediated by the enzyme terminal deoxynucleotidyl transferase (TdT)(171,221), which is present in the nucleus of all immature B and T cells (222,223). TdT is able to add nucleotides to 3´ ends of DNA breakpoints without need for a template (222). The strong link between TdT and N region insertion has been further established by experiments in which TdT$^-$ transformed cells appeared to produce N regions at junction sites upon artificial introduction of active TdT genes via retroviral expression vectors (224).

Insertion of N regions at joining sites can increase the diversity of antigen receptors. This N region diversity occurs in rearranged IgH genes, but is limited or absent in rearranged IgL genes (171,221,224). The junctional diversity has also been found in rearranged TcR genes (87,201-204,211,225-230). Especially in TcR-γ genes and TcR-δ genes large N regions have been identified (Table 1)(87,211-213,228-230). As expected, no N region diversity could be demonstrated in the rearranged TcR-δ genes of TdT$^-$ murine fetal thymocytes (211).

The size of the N region can vary from a few nucleotides up to nine or more (171,211,221, 225,230). If one assumes a random use of the four different nucleotides, then the diversity per junction site in case of insertion of one to six nucleotides is : $4^6 + 4^5 + 4^4 + 4^3 + 4^2 + 4^1 = 5460$ (211). Of course such calculations should be corrected for the probability that the triplet reading frame of the junctional sequences is maintained (one third of the insertions). Further correction should include the chance of termination codons (10% of the in-frame joins) as well as the chance of triplets that code for identical amino acids (about one third of the codons) (211). Still the potential diversity of N regions is very high and can therefore dramatically increase the combinatorial diversity (155,211).

Rough estimations suggest that the N region diversity can increase the repertoire of Ig molecules at least 10^4 fold to more than 10^{10}. The TcR-$\alpha\beta$ repertoire may in this way increase 10^6 fold to $> 10^{12}$. The N region diversity of TcR-δ is extremely high ($> 10^9$ fold increase) due to the relatively high number of inserted nucleotides per junction site and to the variable number of junction sites in the TcR-δ genes (155,211). Therefore it seems that a major part of the variability of the TcR-δ chain and the other TcR chains is concentrated in the V(-D)-J junctional region. This has important implications for our understanding of antigen recognition, since this junctional region of TcR molecules corresponds to the CDR3 of Ig molecules and probably directly interacts with the antigen (155-157).

REARRANGEMENT OF IMMUNOGLOBULIN GENES AND T CELL RECEPTOR GENES DURING LYMPHOID DIFFERENTIATION

Studies in mouse and man have demonstrated that the processes of Ig and TcR gene rearrangement occur in an ordered manner during differentiation.

Immunoglobulin gene rearrangement during B cell differentiation

Studies on transformed murine precursor B cells (231,232) and human precursor-B-cell acute lymphoblastic leukemia (precursor-B-ALL) as malignant counterparts of normal precursor B cells (178-180,233-237) have demonstrated that the IgH genes rearrange early during B cell differentiation and prior to IgL gene rearrangement. First D to JH rearrangement occurs, followed by V to D-JH rearrangement (232). Subsequently the Igκ genes rearrange as demonstrated by studies on precursor-B-ALL, B cell chronic lymphocytic leukemias (B-CLL) and other mature B cell malignancies (178,179,233-235,238-241). Functional rearrangement and transcription of IgH and Igκ genes will result in expression of IgH/κ molecules at the cell surface (238-242).

If the Igκ gene rearrangement is not functional on any of the two alleles, the Igλ genes will rearrange (238-242). Transcription of functionally rearranged Igλ genes in the presence of functional IgH gene transcripts will result in surface membrane expression of IgH/λ molecules. Interestingly, in the majority of Igλ expressing B cells the Cκ genes and in a part of them also the Jκ genes are deleted on both alleles (238,239,241,242). This deletion is mediated via a rearrangement process in which a κ deleting element (Kde) replaces the Cκ gene (60% of cases) or the whole Jκ-Cκ gene segment (40% of cases)(243,244). Like in V(-D)-J rearrangements, also in this rearrangement heptamer-nonamer sequences are involved (244,245). The deletion of κ genes in Igλ expressing B cells may serve as a protective mechanism to eliminate aberrant transcripts and protein fragments that might interfere with the synthesis and assembly of effective Ig molecules (243,244).

A second type of rearrangement in the IgH genes is the class switch process, in which one of the switch sequences in front of the CH genes rearrange to the switch sequences in front of the Cμ-Cδ gene segment (18,25,39). The various Ig gene rearrangements are summarized in Figure 11 (246).

T cell receptor gene rearrangement during T cell differentiation

Studies on murine fetal thymocytes and hybrids derived from murine fetal thymocytes indicate that during T cell ontogeny the TcR-δ genes rearrange at day 14 of gestation (247,248), followed by rearrangement of the TcR-γ and TcR-β genes during day 14 to 17 (248-250). Fully rearranged TcR-β genes are present from day 16 on, when probably also TcR-α genes start to rearrange (249). This hierarchy of TcR gene rearrangements is supported by RNA studies, which indicate that high levels of TcR-γ gene transcripts and probably also TcR-δ gene transcripts are present at day 14 and day 15, but subsequently decline (247,251,252). Mature

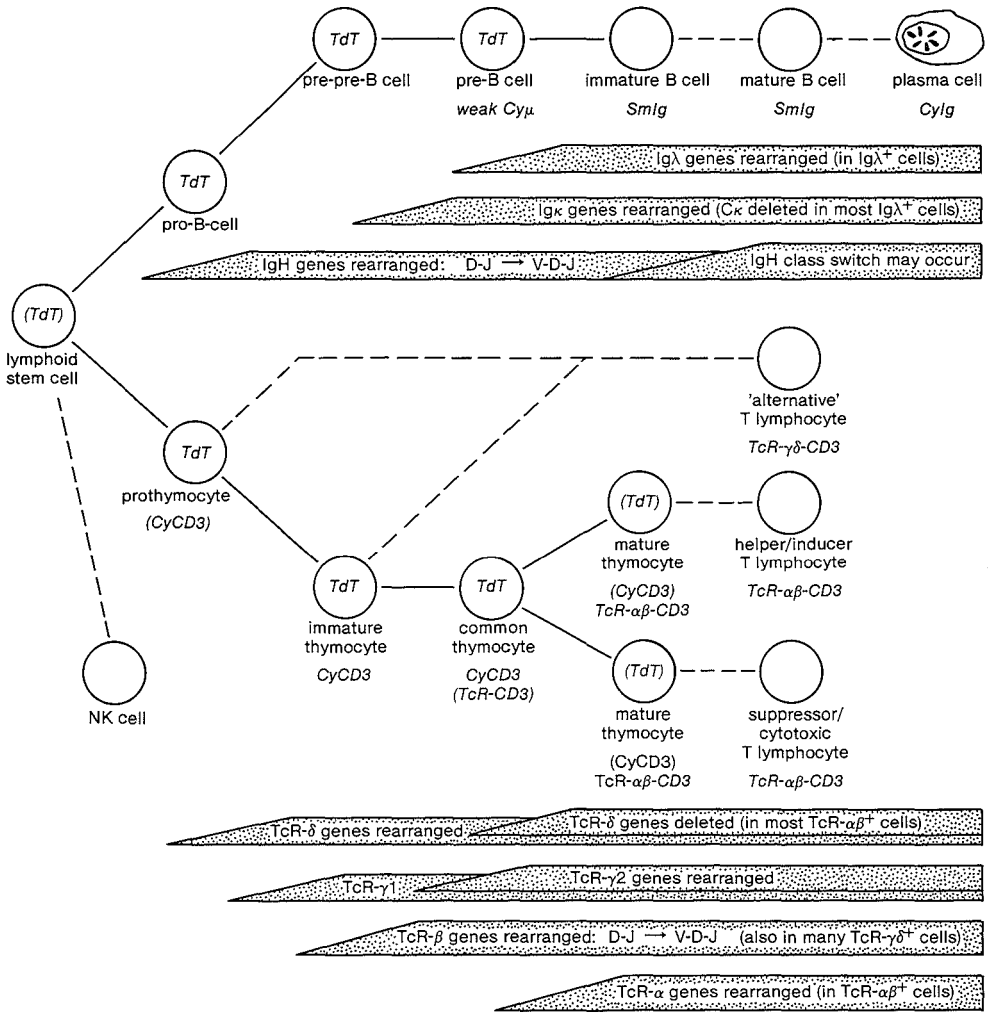


Figure 11. Hypothetical scheme of lymphoid differentiation, summarizing the available data about the ordered rearrangement of the Ig and TcR genes, as well as the expression of TdT, Ig molecules and TcR molecules. The horizontal bars represent the rearrangement of the Ig genes (IgH, Igκ and Igλ) and TcR genes (TcR-α, TcR-β, TcR-γ and TcR-δ). The expression of TdT, weak Cyμ, Smlg, Cylg, CyCD3 and the TcR-CD3 complex (TcR-αβ or TcR-γδ) is given in italics (c.f. ref. 246).

TcR-β gene transcripts are present from day 16 on and TcR-α gene transcripts can be detected from day 17 on (251,252). These data are in line with the appearance of TcR-γδ expressing thymocytes as early as day 15, while TcR-αβ expressing cells are present in increasing numbers from day 17 on (253-255).

The murine ontogenic data (247-255) seem to reflect normal T cell differentiation as studied by analysis of human T cell malignancies and T cell clones (180-183,213,256-264). Especially immature T cell acute lymphoblastic leukemias (T-ALL) without expression of a TcR-CD3

complex at the cell surface appeared to be informative (180-183,213,256-264). The majority of these immature $CD3^-$ T-ALL had rearranged TcR-γ and TcR-β genes (180-183,213,256-261) as well as rearranged TcR-δ genes (213,262-264). A few $CD3^-$ T-ALL had germline γ and β genes (182,213,256,257,259-261) and recently some immature T-ALL with rearranged TcR-δ genes but germline γ and β genes have been found (213,263). This suggests that TcR-δ gene rearrangement occurs before rearrangement of the TcR-γ and TcR-β genes. $CD3^-$ T-ALL and $CD3^-$ T-NHL with rearranged TcR-γ genes but germline TcR-β genes are rare (257,265,266). This suggests that TcR-γ and TcR-β genes rearrange at virtually the same early T cell differentiation stage. It also explains why most mature TcR-$\gamma\delta$ expressing cells have rearranged TcR-β genes (94,264,267). Interestingly, TcR-$\gamma\delta^+$ cells which use γ1 genes often have germline TcR-β genes or incompletely rearranged TcR-β genes, while TcR-$\gamma\delta^+$ cells which use γ2 genes generally have completely rearranged TcR-β genes (94,264,267). Most $CD3^-$ and $CD3^+$ T cell malignancies have rearranged their TcR-γ2 genes and have consequently deleted their TcR-γ1 genes (180,256,259,260,264,268,269). The majority of $CD3^+$ T cell malignancies probably express TcR-$\alpha\beta$ as determined by the presence of TcR-α gene rearrangements and/or TcR-α mRNA (181-183,259, 260) or by reactivity with an anti-TcR-$\alpha\beta$ antibody (260,264). Most of the TcR-$\alpha\beta^+$ malignancies have deleted both TcR-δ gene alleles, since the TcR-δ genes are located between the Vα and Jα gene segments (Figure 4)(70,83,212,213,262-264). In a part of the T cells one TcR-δ gene allele is deleted, while the other allele is still present (70,213,247,262--264).

All these data are compatible with a model of T cell differentiation in which the TcR-δ genes rearrange first, immediately followed by rearrangement of the TcR-γ genes and in most cells also rearrangement of the TcR-β genes. Probably the TcR-γ gene rearrangement first involves the γ1 locus and if this rearrangement is non-functional, rearrangement to the γ2 locus occurs (94,267). Transcription of functional TcR-γ and TcR-δ genes results in TcR-$\gamma\delta$ expressing cells. If the TcR-γ and/or TcR-δ gene rearrangements are not functional or incomplete, the involved T cells remain TcR-$CD3^-$ and will probably be eliminated. On the other hand, these T cells or newly formed precursor T cells may delete one or both TcR-δ genes and rearrange their TcR-β and TcR-α genes. There is evidence that a specific deletion of the D, J and C segments of the TcR-δ genes occurs before rearrangement of the TcR-α genes (213,270-272). These two separate consecutive steps are therefore comparable to the deletion of Igκ gene segments in B cells, which rearrange their Igλ genes (238,242). Functional rearrangement of the TcR-α and TcR-β genes results in TcR-$\alpha\beta$ expressing cells. Non-functional rearrangements will probably lead to the cell death.

It is not yet clear whether the TcR-$\gamma\delta$ differentiation pathway is separate from the TcR-$\alpha\beta$ pathway or whether the TcR-$\gamma\delta^+$ cells develop prior to the TcR-$\alpha\beta^+$ cells in the same linear differentiation pathway. The presence of TcR-β gene rearrangements in TcR-$\gamma\delta^+$ cells (94,264,267) and the presence of TcR-γ gene rearrangements (generally in the γ2 locus) in TcR-$\alpha\beta^+$ cells (180,256,259,260,264,268,269) are in favor of a linear differentiation pathway (94,267). However, a functional rearrangement of the TcR-δ genes on the one hand and the specific deletion of the Dδ-Jδ-Cδ gene segment on the other hand may represent regulation events, which separate the TcR-$\gamma\delta$ pathway from the TcR-$\alpha\beta$ pathway (213,271). The recent detection of circular excision products with germline Dδ, Jδ and Cδ gene segments in the thymus supports this hypothesis (273). This would imply that the rearrangement/deletion

processes in the TcR-δ genes play a crucial role in the separation of the $\gamma\delta$ and $\alpha\beta$ pathways. In this case the occurrence of TcR-γ gene rearrangements in TcR-$\alpha\beta^+$ cells and TcR-β gene rearrangements in TcR-$\gamma\delta^+$ cells may be explained by activity of a "common TcR gene recombinase". The various processes in the hierarchy of TcR gene rearrangements are summarized in Figure 11. Additional aspects concerning the divergence of the $\gamma\delta$ and $\alpha\beta$ pathways are discussed in Chapter 4.1.

The unique structure of the TcR-α/δ locus may not only ensure a controlled hierarchy of gene rearrangements, but may also ensure the mutually exclusive expression of TcR-α and TcR-δ genes to avoid dual receptor expression (70,264). In addition, the deletion of the Dδ-Jδ-Cδ segment before the rearrangement of the TcR-α genes prevents that Dδ and Jδ gene segments, which provide the major source of TcR-δ gene diversity, are used in conjunction with TcR-α (271).

It is not yet known to what extent all TcR gene rearrangements are thymus dependent. Interestingly, in nude mice as well as in athymic man, i.e. patients with a complete DiGeorge syndrome or a classical type of severe combined immunodeficiency, TcR-$\gamma\delta^+$ cells are present, while TcR-$\alpha\beta^+$ cells are virtually absent (274-276). This suggests that TcR-$\gamma\delta^+$ cells are still able to develop in athymic species, while the development of TcR-$\alpha\beta^+$ cells is highly thymus dependent (276).

Natural killer cells (NK cells) are generally considered as a subpopulation of T cells since most of them express the pan-T cell markers CD2 and CD7 in addition to the CD16 antigen (Fc receptor for IgG) and the CD56 antigen (Leu-19 antigen)(277-280). However, several reports indicate that functional NK cells do not express a TcR-CD3 complex at the cell surface (277-280) and have all TcR genes tested in germline configuration (281-286). Therefore NK cells are probably derived from precursor T cells, which did not (yet) rearrange their TcR genes (Figure 11).

Relationship between protein expression and gene rearrangement

During B cell differentiation the results of functional IgH gene rearrangement can be seen at the pre-B cell stage, i.e. weak cytoplasmic expression of the IgH chain μ (weak Cyμ)(287). SmIg expression does not occur until functional rearrangement of both IgH and IgL genes has occurred (Figure 11). To a certain extent this relationship between Ig protein chain expression and Ig gene rearrangement is illustrated by the phenotypic and genotypic characteristics of precursor-B-ALL (178-180,233-237). While virtually all precursor-B-ALL (null ALL, common ALL and pre-B-ALL) have rearranged IgH genes, the IgL gene rearrangements are found in only a part of the common ALL (pre-pre-B cell) and pre-B-ALL (pre-B cell)(178-180,233-237).

Analogous to SmIg expression during B cell differentiation, the expression of the TcR-CD3 complex at the cell surface of T cells only occurs after functional rearrangement of TcR-γ and TcR-δ genes or functional rearrangement of TcR-α and TcR-β genes. Expression of the TcR-CD3 complex at the cell surface is preceded by cytoplasmic expression of the CD3 protein chains (CyCD3) during the early stages of T cell differentiation (Figure 11)(181,288-290). Therefore CyCD3 staining can be used as a diagnostic marker for immature $CD3^-$ T cell malignancies (181,290-294). Immature normal $CD3^-$ T cells as well as $CD3^-$ T cell malignancies

may also be positive for cytoplasmic TcR-β chains (295-297). It should be remarked that such expression does not prove that these cells are precursors of TcR-$\alpha\beta^+$ cells, since also TcR-$\gamma\delta^+$ cells may contain functional TcR-β gene transcripts (63,64,94,264) and TcR-β protein chains (62).

The nuclear enzyme TdT is expressed by all immature lymphoid cells, but is absent in mature B and T lymphocytes (222,223). This suggests that TdT expression disappears after functional rearrangement of Ig and TcR genes, which is in agreement with the postulate that TdT mediates the insertion of N regions at the joining sites during gene rearrangement (171,221,224). Although during T cell differentiation coexpression of TdT and the TcR-CD3 complex at the cell surface is found in the thymus, such coexpression of SmIg and TdT during normal B cell differentiation is not found (298-300). Along with these findings, only a few TdT^+/$SmIg^+$ B-ALL have been reported (300-302), while large series of TdT^+/$CD3^+$ T-ALL have been described (181-183,259). This discrepancy may be related to the virtual absence of N regions in rearranged IgL genes (171,221,224), while N region diversity is present in all rearranged TcR genes (201-204,225-230). It suggests that during B cell differentiation TdT activity decreases as soon as IgH gene rearrangement is completed, resulting in the absence of TdT when SmIg is started to be expressed.

DIFFERENCES BETWEEN IMMUNOGLOBULIN AND T CELL RECEPTOR GENES AND THEIR PROTEIN PRODUCTS

The genes which code for the various Ig and TcR chains have similar germline organizations and undergo similar gene rearrangement processes (Figures 2 and 4)(13-15). This is reflected at the protein level where the primary, secondary and tertiary structure of the protein chains have a high degree of homology (Figure 1)(reviewed in ref. 303 and 304). Despite these similarities several major differences exist between the Ig and TcR molecules and their function (Table 2).

We shall first discuss the relatedness between Ig and TcR molecules. Subsequently we shall consider several differences between Ig and TcR genes and molecules.

Immunoglobulin gene superfamily

The structure of the Ig and TcR molecules are related to each other as well as to other molecules which are involved in specific immune recognition, such as the major histocompatibility complex (MHC) molecules of class I and class II and the T cell accessory molecules CD4 and CD8 (Figure 12)(303-306). These similarities are not restricted to the protein structure, but are also present at the level of gene organization, suggesting that the encoding genes have a common evolutionary origin (306). These closely related genes belong to the so-called Ig gene superfamily (305, 306).

Hood et al. defined the Ig gene superfamily as a set of multigene families and single-copy genes related by sequence (implying common ancestry), but not necessarily related in function (306). Members of this superfamily code for protein chains with domains which are homolo-

TABLE 2. Differences between the antigen specific receptors of B and T lymphocytes.

	B lymphocytes	T lymphocytes
Basic structure of the receptor		
- number of protein chains per molecule	4	2
- number of constant domains per chain	1-4[a]	1
- number of antigen binding/recognition sites per molecule	2	1
- close association with other molecules	–	+ (CD3 molecules)
Antigen recognition/binding		
- HLA-restricted antigen recognition	–	+[b]
- antigen-induced somatic mutations	+	–
- affinity of the receptor	high (10^8-10^{13})	low ($<10^6$)
Changes of the receptor during maturation		
- secretion of receptor molecules	+	–
- isotype switching	+	–

a. The IgL chains contain one constant domain, while the IgH chain contains three constant domains (δ, γ and α) or four constant domains (μ and ϵ) (Figure 3).
b. The antigen recognition is MHC-class I restricted in case of TcR-$\alpha\beta^+$/CD8$^+$ cells and HLA-class II restricted in case of TcR-$\alpha\beta^+$/CD4$^+$ cells. TcR-$\gamma\delta^+$ T lymphocytes probably also recognize antigen in association with MHC or MHC-like molecules.

gous to the Ig chain domains. These so-called Ig homology units are composed of 100 to 110 amino acids and have a centrally placed disulfide bridge, which spans about 65 amino acids and stabilizes the secondary and tertiary structure of the homology unit (303-306). Two main types of homology units can be recognized: the V and C homology units, named after the respective domains of the Ig molecules (c.f. Figure 12)(303-306). The degree of amino acid sequence similarities between homology units of distinct families varies from about 15 to 40% (306), whilethe degree of homology within a family is much higher (303). At the DNA level each homology unit is usually encoded by a separate exon (306). This demonstrates the striking correlation between the distinct structural features of these proteins and the exon-intron organization of their encoding genes (306).

Homology to Ig units is also found in molecules which are not directly involved in the immune system. Some of these molecules have no known function (Thy-1, MRC OX-2, α1B-glycoprotein), while others interact with homologous molecules either within the immune system (e.g. the CD58 molecule (LFA-3) and Fc receptors) or in different systems (e.g. the CD56 molecule (NCAM) and MAG/Hcp3)(303-308).

Nine multigene families and at least twenty-five single-gene families have been included in the Ig gene superfamily so far. The multigene families are the Ig genes, the TcR genes and the MHC-class I and class II genes. The single-gene families include the genes for the CD1 molecules, the T cell antigens (CD2, CD3, CD4, CD7, CD8 and CD28), the hematopoietic-endothelium antigens (CD58 molecule and MRC OX-45), the brain-lymphoid antigens (Thy-1 and MRC OX-2), Fc receptors, neural molecules (e.g. CD56 molecule and MAG/Hcp3), growth factor receptors (PDGF receptor and CSF1 receptor) and non-cell surface molecules (e.g. α1B-glycoprotein)(303-308).

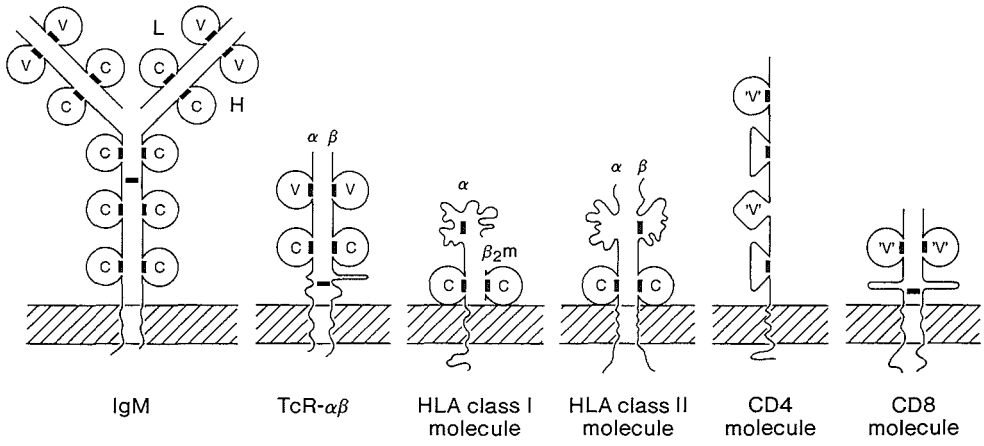


Figure 12. Schematic diagram of several members of the Ig gene superfamily i.e. IgM molecule, TcR-$\alpha\beta$ molecule, HLA-class I molecule, HLA-class II molecule, CD4 molecule and CD8 molecule. V: variable domain, C: constant domain, β2m: β2-microglobulin, 'V': V-like domain.

Thus the members of the Ig gene superfamily extend beyond the immune system. This suggests that the Ig homology unit has played a central role in cell-cell interaction during evolution (307). Therefore investigation of structure and function of related systems in lower organisms may provide clues about the primordial function of the members of the Ig gene superfamily (303).

Major histocompatibility complex-restricted antigen recognition

One of the major differences between Ig and TcR molecules is that the TcR of most T lymphocytes is able to recognize the corresponding antigen only if this is associated with an MHC molecule on the surface membrane of another cell (309-312). In contrast, Ig molecules can bind to both free and membrane-bound antigens. After recognition of the antigen-MHC complex T lymphocytes will be activated and will exhibit their specific regulatory or cytotoxic functions. This phenomenon is called MHC-restricted antigen recognition and has been demonstrated to occur in case of TcR-$\alpha\beta^+$ T lymphocytes (309-312). TcR-$\gamma\delta^+$ T lymphocytes probably also recognize antigen in association with MHC or MHC-like molecules (313-315).

The mature TcR-$\alpha\beta^+$ T lymphocytes express either CD4 or CD8 molecules. The majority of the $CD4^+$ T lymphocytes exhibit helper/inducer functions, while most $CD8^+$ T lymphocytes exhibit cytotoxic/suppressor functions (316,317). However, a subset of $CD4^+$ T lymphocytes can also display cytolytic activity (318,319). There appears to exist a strong correlation between CD4 expression and MHC-class II-restricted antigen recognition and between CD8 expression and MHC-class I-restricted antigen recognition (309-312,316,317). This implies that $CD4^+$ T lymphocytes can only be activated if the relevant antigen is presented by MHC-class II expressing cells, such as B lymphocytes, monocytes and most macrophages. It is assumed

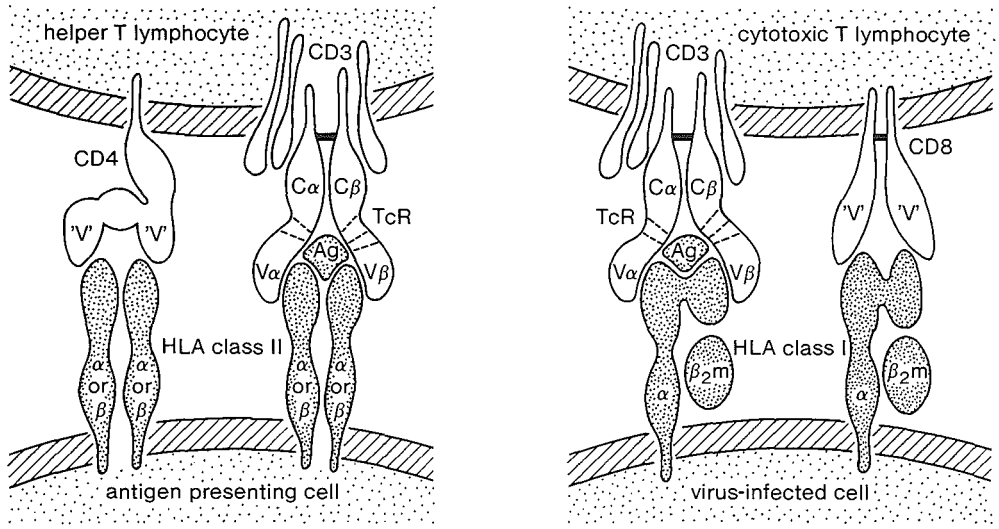


Figure 13. Hypothetical schemes of MHC-restricted antigen recognition by T lymphocytes. **A**: $CD4^+$ T lymphocytes recognize antigens presented by HLA-class II molecules. **B**: $CD8^+$ T lymphocytes recognize antigens presented by HLA-class I molecules. In these figures the junctional regions (CDR3-equivalent region) of the TcR-$\alpha\beta$ molecules contact the antigen (Ag), while the distal parts of the V domains (containing the CDR1 and CDR2-equivalent regions) contact the polymorphic domains of the MHC molecules (155,157,332). The binding of the CD4 and CD8 molecules to the HLA-class II and HLA-class I molecules, respectively, is depicted as occurring in basically the same way as suggested for the TcR-$\alpha\beta$ molecules (155,311). The latter probably implies that TcR and CD4 or CD8 interact with separate MHC molecules.

that $CD8^+$ T lymphocytes can be activated by each nucleated cell, since they all express MHC-class I molecules.

Several reports indicate that the antigen recognized by the TcR-$\alpha\beta^+$ T lymphocytes is a peptide fragment of the antigen (presumably derived from intracellular processing), which is bound to a specific site of the MHC molecule (320-322). The recent description of the crystal structure of a human MHC-class I molecule has demonstrated that the binding site for the peptide fragment is a cleft on the outer surface of the MHC molecule, located between two polymorphic domains (323,324). The structurally related MHC-class II molecules are predicted to contain a similar peptide binding site (325).

It has been suggested that the Vα domains of the TcR are associated with recognition of the antigenic peptide fragment, while the use of specific Vβ domains correlates with the recognition of the polymorphic part of certain MHC molecules (326-329). However, such a correlation between V gene usage and antigen or MHC recognition has not been found in all studies (330-332). Alternatively it has been suggested that the CDR3-equivalent regions of both V domains contact the antigen fragment, while the distal parts of the V domains (containing CDR1 and CDR2-equivalent regions) contact the polymorphic domains of the MHC molecules (Figure 13) (155,157,332).

The T cell accessory molecules CD4 and CD8 probably play a role in the interaction between the T lymphocyte and the antigen-presenting cell or target cell (311,317,333-335). It

has been suggested that the CD4 and CD8 molecules in fact are receptors for MHC-class II and MHC-class I molecules, respectively, and that these T cell accessory molecules enhance in this way the contact between the TcR and the antigen-presenting MHC molecules (333-343). It is not fully understood how the CD4 and CD8 molecules interact with the MHC molecules. There is some evidence that CD4 and CD8 molecules are physically associated with the TcR-CD3 complex and therefore probably interact with the constant (non-polymorphic) domain of the same MHC molecule which presents antigen to the TcR (344-346). On the other hand, it might well be that the accessory molecules and the TcR interact with separate MHC molecules, which allows interaction of the V-like domains of the CD4 and CD8 molecules with (non-polymorphic epitopes of) the polymorphic domains of the MHC molecules (Figure 13)(155,311). Future studies will elucidate the precise nature of these interactions.

Somatic mutation, antibody diversity and antibody affinity

The antibody repertoire of virgin B cells is based on the combinatorial and junctional repertoire of the Ig genes, acquired during the antigen-independent stages of B cell differentiation (13-15,149). During the immune response B cells can change the antigen-binding properties of the antibodies, resulting in the production of antibodies with higher affinities (14,347,348). Several studies on the development of the immune response against simple antigens (e.g. oxazolone, *p*-azophenylarsonate and hydroxy-nitro-phenylacetyl) have demonstrated that these changes are caused by molecular events in the V regions, so-called somatic mutations (349-352). Somatic mutations are point mutations in the nucleotide sequence of V regions, preferentially in the CDR1 and CDR2 encoding sequences (14,348,349). The mutations do not only occur in VH genes, but also in VL genes (14,348-352). The frequency of somatic mutation in the V gene segments during the immune response is estimated to be about 10^{-3}/base pair/cell division (353-356), more than a thousand times higher than the mutation frequency in other genes (353).

Somatic mutations do not occur in virgin B cells, as shown in non-immune mice, but arise only after antigenic stimulation (351,357-359). Some reports indicate that the mutation mechanism is not continuously active during the immune response, but seems to concentrate in the mid to late phase of the primary response (356,359). It has been postulated that the mutations especially occur in B cells of the memory pathway (359). Reexposure to the antigen leads to activation of only those memory B cells, which carry advantageous antibody mutants, i.e. antibodies with a higher affinity (348,359). It was demonstrated that the intraclonal antibody affinity can increase more than 15 fold (360). Increase in antibody affinity could also be obtained via specific V region mutations by use of oligonucleotide-directed mutagenesis (361).

The somatic mutation-mediated maturation and clonal expansion of the immune response is antigen driven (348-360) and cannot be mimicked *in vitro* with polyclonal mitogens (362). Interestingly, anti-idiotype (Id) antibodies can select for B cells which changed their Id via somatic mutation. This became evident in clinical studies concerning the *in vivo* treatment of B-NHL with anti-Id antibodies (363,364). Several B-NHL patients only had a partial response to the antibody therapy due to a strong selection and growth of Id^- B-NHL cells (363,364). In some follicular lymphomas Id variants could already be detected at diagnosis (365). In an

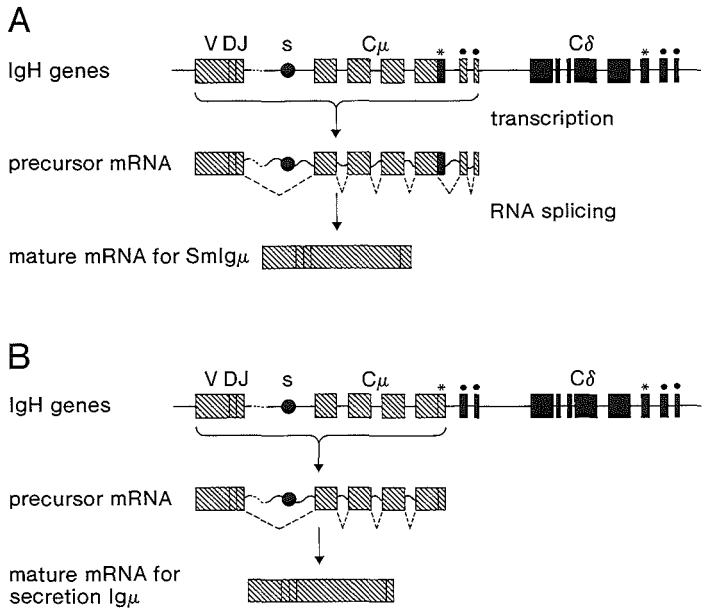


Figure 14. Schematic diagram of RNA splicing processes for the production of membrane bound Ig molecules or secretory Ig molecules. Membrane bound Ig molecules and secretory Ig molecules differ in their carboxy-terminal part, which is encoded by secretion sequences or membrane sequences. The terminal secretion sequences (*) are generally contiguous with the final IgH-C region exon, while the membrane sequences (•) are located in one or more exons downstream of the last CH exon. Mature forms of secretion mRNA and membrane mRNA are produced via a process of RNA splicing (c.f. ref. 371-374).

extensive study on one patient it was proven that many point mutations had occurred in the various VH gene segments of the lymphoma cells (366).

So far, no evidence has been produced indicating somatic mutation in the V segments of the TcR-α and TcR-β genes (201,367-370).

Surface membrane bound Ig molecules and secreted Ig molecules

The Ig molecules on the cell membrane (SmIg) are the antigen-specific receptors of B lymphocytes, while the soluble Ig molecules in serum, tissue fluids and mucosal layers are the antibodies which play an important role in the neutralization and opsonization of micro-organisms and other antigens. The serum antibodies (IgM, IgG and IgA) are mainly secreted by plasma cells in the bone marrow, while the antibodies in the mucosal layers (especially IgA) are produced locally. In plasma cells high amounts of Ig molecules are synthesized and accumulated intracellularly. These are detectable as 'cytoplasmic Ig molecules' (CyIg).

The difference between the two forms of Ig molecules is located in the carboxy-terminal part of the IgH chain. The genetic basis for this difference resides in the exon organization of the CH gene segments. The terminal secretion sequences are generally contiguous with the

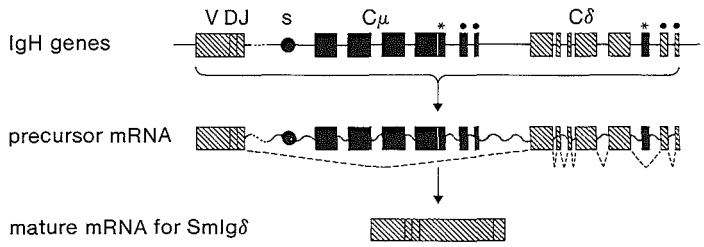


Figure 15. Schematic diagram of the production of mature mRNA for the IgH chain of IgD molecules. Mature mRNA for the IgH chain of IgM or IgD is produced via alternative processing of precursor mRNA, resulting in splicing of the V-D-J segment to Cμ gene segments or to Cδ gene segments (377,378).

final IgH-C region exon, while the surface membrane binding sequences are located in one or more exons downstream of the last CH exon (371,372). Regulation of the production of SmIg versus secretory CyIg most probably occurs at the mRNA level via a process of RNA splicing (Figure 14)(371-374).

So far, there are no reports on the occurrence of secreted forms of TcR. Also at the DNA level no evidence has been found for the existence of secretory forms of TcR molecules.

IgH class switching

Most B lymphocytes in the peripheral blood express IgM and IgD on their cell surface, while a smaller part expresses IgG or IgA. In lymph nodes and spleen a higher frequency of IgG and IgA bearing lymphocytes is found. Coexpression of multiple IgH classes may occur in B lymphocytes (375), but plasma cells produce only one IgH chain of only one (sub)class (376).

Coexpression of IgM and IgD molecules on the cell surface of B lymphocytes can be mediated by alternative RNA processing (377,378). This results in splicing of the VDJ gene segment to the Cμ gene segments or to the Cδ gene segments (Figure 15).

Early studies have indicated that IgH class switching from IgM and IgD to IgG or IgA can be mediated via a second type of Ig gene rearrangement, which replaces the Cμ gene by the appropriate CH gene, thereby deleting all intervening sequences (379,380). The recombination sites of this rearrangement appeared to contain repetitive sequences, the so-called switch (S) regions, which are located at the 5′ side of each CH gene except for the Cδ gene (Figures 15 and 16)(38,39,381,382). These S regions are probably directly or indirectly involved in the IgH class switch rearrangement, which is therefore called S-S recombination (39). Although the precise mechanism of the S-S recombination is unknown, recent studies indicate that the process may occur by looping-out and deletion (Figure 16)(383).

Several studies indicate that IgH class switch or coexpression of IgM and IgG or IgM and IgA may occur without any detectable S-S recombination (384-386). It was concluded that this type of IgH isotype expression is accomplished by alternative RNA processing of large (up to 180 kb) transcripts that span the IgH-C region locus (384-386).

The combined data have led to the hypothesis that IgH class switching may be a two-step

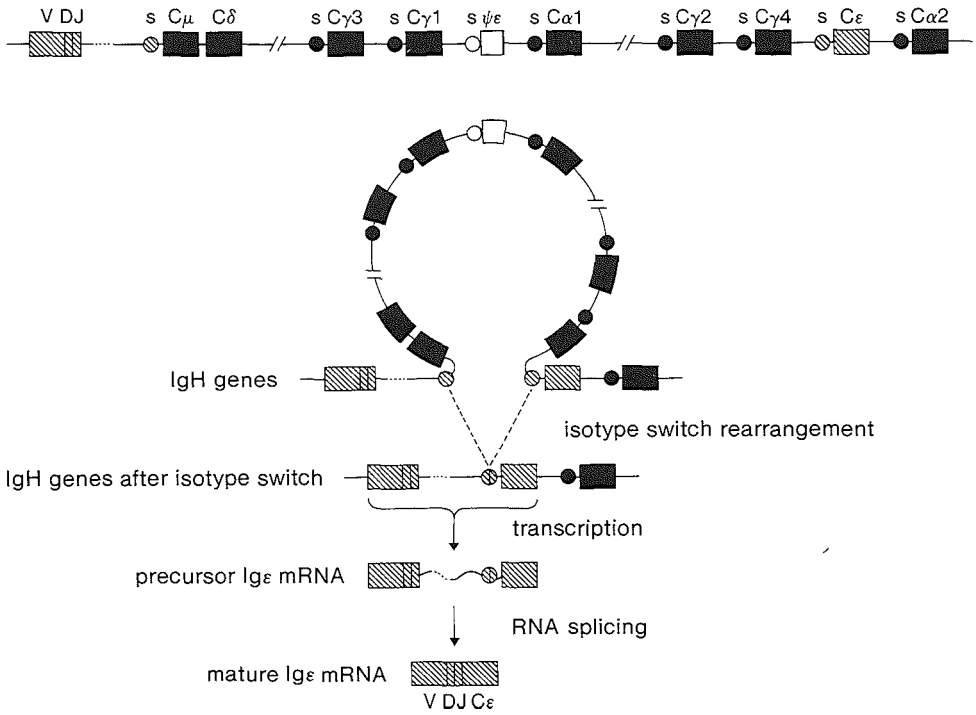


Figure 16. Schematic diagram of IgH class switch via S-S recombination. During IgH class switch the S regions play an important role in the recombination, which probably occurs by looping-out and deletion of all intervening DNA sequences (39,383).

process. First formation of large transcripts and expression of the various IgH isotypes occurs by differential RNA splicing. Later, during the course of B cell activation and maturation, S-S recombination represents the final step of IgH class switching (387,388). Recent evidence indicates that this two-step IgH class switch process can be regulated by interleukins such as γ-interferon, interleukin-4 and interleukin-5 (389-391). Interestingly there appears to be no correlation between the IgH class switching on the productive IgH gene allele and the class switching on the non-productive allele (392-394). Therefore, it has to be concluded that interleukins only regulate the class switching of the productive IgH gene allele.

While IgH class switching is a frequent event in B lymphocytes, no evidence has been found for switching from one type of TcR to another type of TcR in T lymphocytes. Although it has been found that TcR-$\gamma\delta^+$ cells develop earlier than TcR-$\alpha\beta^+$ cells during murine T cell ontogeny (247-255), there is no indication that TcR-$\gamma\delta^+$ cells can develop into TcR-$\alpha\beta^+$ cells or that TcR-$\gamma1\delta^+$ cells can develop into TcR-$\gamma2\delta^+$ cells.

CONCLUSION

B and T lymphocytes play a central role in the immune system due to their ability to

specifically recognize antigens via their SmIg and TcR molecules, respectively. The structure of the Ig and TcR genes, the gene rearrangement processes and the insertion of nucleotides in the junctional regions form the basis of the diversity of these antigen-specific receptors of lymphocytes. Despite the similarities between Ig and TcR genes/molecules, several major differences exist between B and T lymphocytes, such as the MHC-restricted antigen recognition of T lymphocytes and the somatic mutations and Ig class switch of B lymphocytes. These differences makes B and T lymphocytes to different, but complementary immune cells.

ACKNOWLEDGMENTS. We gratefully acknowledge Professor Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Mr. T.M. van Os (Department of Immunology, Erasmus University, Rotterdam) and Mr. C. van Dijk (Interpoint, Hapert) for excellent assistance in the preparation of the figures and Mrs. J. de Goey-van Dooren, Ms. E.P. Schenkel and Ms. H.J. de Jager for their secretarial assistance.

REFERENCES

1. Nossal GJV. The basic components of the immune system. N Engl J Med 1987;316:1320-5.
2. Cooper MD. B lymphocytes: normal development and function. N Engl J Med 1987;317:1452-6.
3. Marrack P, Kappler J. The T cell receptor. Science 1987;238:1073-9.
4. Royer HD, Reinherz EL. T lymphocytes: ontogeny, function, and relevance to clinical disorders. N Engl J Med 1987;317:1136-42.
5. Burton DR. Structure and function of antibodies. In: Calabi F, Neuberger MS, eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier Science Publishers, 1987;1-50.
6. Meuer SC, Acuto O, Hercend T, Schlossman SF, Reinherz EL. The human T-cell receptor. Annu Rev !mmunol 1984;2:23-50.
7. Allison JP, Lanier LL. Structure, function, and serology of the T-cell antigen receptor complex. Annu Rev Immunol 1987;5:503-40.
8. Oettgen HC, Kappler J, Tax WJM, Terhorst C. Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. J Biol Chem 1984;259:12039-48.
9. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The δ- and ϵ-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature 1984;312:455-8.
10. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu Rev Immunol 1988;6:629-62.
11. Weissman AM, Samelson LE, Klausner RD. A new subunit of the human T-cell antigen receptor complex. Nature 1986;324:480-2.
12. Weissman AM, Hou D, Orloff DG, Modi WS, Seuanez H, O'Brien SJ, Klausner RD. Molecular cloning and chromosomal localization of the human T-cell receptor ζ chain: distinction from the molecular CD3 complex. Proc Natl Acad Sci USA 1988;85:9709-13.
13. Leder P. The genetics of antibody diversity. Sci Am 1982;246(5):102-15.
14. Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575-81.
15. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
16. Tonegawa S. Reiteration frequency of immunoglobulin light chain genes: further evidence for somatic generation of antibody diversity. Proc Natl Acad Sci USA 1976;73:203-7.
17. Tonegawa S, Steinberg C. Too many chains - too few genes. In: Cunningham AJ, ed. The generation of antibody diversity: a new look. London: Academic Press, 1976:175-82.
18. Shimizu A, Honjo T. Immunoglobulin class switching. Cell 1984;36:801-3.
19. Krawinkel U, Radbruch A. Immunoglobulin heavy chain class switching. In: Calabi F, Neuberger MS, eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier Science Publishers, 1987:135-51.
20. Berman JE, Mellis SJ, Pollock R, Smith CL, Suh H, Heinke B, Kowal C, Surti U, Chess L, Cantor CR, Alt

FW. Content and organization of the human Ig VH locus: definition of three new VH families and linkage to the Ig CH locus. EMBO J 1988;7:727-38.
21. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin μ locus: characterization of embryonic and rearranged J and D genes. Cell 1981;27:583-91.
22. Matsuda F, Lee KH, Nakai S, Sato T, Kodaira M, Zong SQ, Ohno H, Fukuhara S, Honjo T. Dispersed localization of D segments in the human immunoglobulin heavy-chain locus. EMBO J 1988;7:1047-51.
23. Buluwela L, Albertson DG, Sherrington P, Rabbitts PH, Spurr N, Rabbitts TH. The use of chromosomal translocations to study human immunoglobulin gene organization: mapping DH segments within 35 kb of the Cμ gene and identification of a new DH locus. EMBO J 1988;7:2003-10.
24. Flanagan JG, Rabbitts TH. Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ, ϵ and α genes. Nature 1982;300:709-13.
25. Rabbitts TH, Forster A, Milstein CP. Human immunoglobulin heavy chain genes: evolutionary comparisons of Cμ, Cδ and Cγ genes and associated switch sequences. Nucleic Acids Res 1981;9:4509-24.
26. White MG, Shen AL, Word CJ, Tucker PW, Blattner FR. Human immunoglobulin D: genomic sequence of the delta heavy chain. Science 1985;228:733-7.
27. Ellison JW, Berson BJ, Hood LE. The nucleotide sequence of a human immunoglobulin Cγ1 gene. Nucleic Acids Res 1982;10:4071-9.
28. Ellison J, Hood L. Linkage and sequence homology of two human immunoglobulin γ heavy chain constant region genes. Proc Natl Acad Sci USA 1982;79:1984-8.
29. Krawinkel U, Rabbitts TH. Comparison of the hinge-coding segments in human immunoglobulin gamma heavy chain genes and the linkage of the gamma 2 and gamma 4 subclass genes. EMBO J 1982;1:403-7.
30. Takahashi N, Ueda S, Obata M, Nikaido T, Nakai S, Honjo T. Structure of human immunoglobulin gamma genes: implications for evolution of a gene family. Cell 1982;29:671-9.
31. Huck S, Fort P, Crawford DH, Lefranc M-P, Lefranc G. Sequence of a human immunoglobulin gamma 3 heavy chain constant region gene: comparison with the human Cγ genes. Nucleic Acids Res 1986;14:1779-89.
32. Flanagan JG, Lefranc M-P, Rabbitts TH. Mechanisms of divergence and convergence of the human immunoglobulin α1 and α2 constant region gene sequences. Cell 1984;36:681-8.
33. Flanagan JG, Rabbitts TH. The sequence of a human immunoglobulin episilon heavy chain constant region gene, and evidence for three non-allelic genes. EMBO J 1982;1:655-60.
34. Nishida Y, Miki T, Hisajima H, Honjo T. Cloning of human immunoglobulin ϵ chain genes: evidence for multiple Cϵ genes. Proc Natl Acad Sci USA 1982;79:3833-7.
35. Max EE, Battey J, Ney R, Krisch I, Leder P. Duplication and deletion in the human immunoglobulin ϵ genes. Cell 1982;29:691-9.
36. Battey J, Max EE, McBride WO, Swan D, Leder P. A processed human immunoglobulin ϵ gene has moved to chromosome 9. Proc Natl Acad Sci USA 1982;79:5956-60.
37. Hisajima H, Nishida Y, Nakai S, Takahashi N, Ueda S, Honjo T. Structure of the human immunoglobulin Cϵ2 gene, a truncated pseudogene: implications for its evolutionary origin. Proc Natl Acad Sci USA 1983;80:2995-9.
38. Kataoka T, Kawakami T, Takahashi N, Honjo T. Rearrangement of immunoglobulin γ1-chain gene and mechanism for heavy-chain class switch. Proc Natl Acad Sci USA 1980;77:919-23.
39. Kataoka T, Miyata T, Honjo T. Repetitive sequences in class-switch recombination regions of immunoglobulin heavy chain genes. Cell 1981;23:357-68.
40. Maki R, Roeder W, Traunecker A, Sidman C, Wabl M, Raschke W, Tonegawa S. The role of DNA rearrangement and alternative RNA processing in the expression of immunoglobulin delta genes. Cell 1981;24:353-65.
41. Knapp MR, Liu C-P, Newell N, Ward RB, Tucker PW, Strober S, Blattner F. Simultaneous expression of immunoglobulin μ and δ heavy chains by a cloned B-cell lymphoma: a single copy of the VH gene is shared by two adjacent CH genes. Proc Natl Acad Sci USA 1982;79:2996-3000.
42. Hieter PA, Max EE, Seidman JG, Maizel JV, Leder P. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 1980;22:197-207.
43. Hieter PA, Maizel JV, Leder P. Evolution of human immunoglobulin κ J region genes. J Biol Chem

1982;257:1516-22.
44. Hieter PA, Hollis GF, Korsmeyer SJ, Waldmann TA, Leder P. Clustered arrangement of immunoglobulin λ constant region genes in man. Nature 1981;294:536-40.
45. Udey JA, Blomberg B. Human λ light chain locus: organization and DNA sequences of three genomic J regions. Immunogenetics 1987;25:63-70.
46. Dariavach P, Lefranc G, Lefranc M-P. Human immunoglobulin Cλ6 gene encodes the $Kern^+Oz^-$ λ chain and Cλ4 and Cλ5 are pseudogenes. Proc Natl Acad Sci USA 1987;84:9074-8.
47. Taub RA, Hollis GF, Hieter PA, Korsmeyer S, Waldmann TA, Leder P. Variable amplification of immunoglobulin λ light-chain genes in human populations. Nature 1983;304:172-4.
48. Chang H, Dmitrovsky E, Hieter PA, Mitchell K, Leder P, Turoczi L, Kirsch IR, Hollis GF. Identification of three new Ig λ-like genes in man. J Exp Med 1986;163:425-35.
49. Hollis GF, Evans RJ, Stafford-Hollis JM, Korsmeyer SJ, McKearn JP. Immunoglobulin λ light-chain-related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. Proc Natl Acad Sci USA 1989;86:5552-6.
50. Bech-Hansen NT, Linsley PS, Cox DW. Restriction fragment length polymorphisms associated with immunoglobulin Cγ genes reveal linkage disequilibrium and genomic organization. Proc Natl Acad Sci USA 1983;80:6952-6.
51. Bottaro A, de Marchi M, Migone N, Carbonara AO. Pulsed-field gel analysis of human immunoglobulin heavy-chain constant region gene deletions reveals the extent of unmapped regions within the locus. Genomics 1989;4:505-8.
52. Hofker MH, Walter MA, Cox DW. Complete physical map of the human immunoglobulin heavy chain constant region gene complex. Proc Natl Acad Sci USA 1989;86:5567-71.
53. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
54. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325:683-8.
55. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 1987;325:689-94.
56. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
57. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.
58. Goodman T, Lefrançois L. Expression of the γ-δ T-cell receptor on intestinal $CD8^+$ intraepithelial lymphocytes. Nature 1988;333:855-8.
59. Tonegawa S, Berns A, Bonneville M, Farr AG, Ishida I, Ito K, Itohara S, Janeway CA Jr, Kanagawa O, Katsuiki M, Kubo R, Lafaille JJ, Mombaerts P, Murphy DB, Nakanishi N, Takagaki Y, Van Kaer L, Verbeek S. Diversity, development, ligands and probable functions of γδ T cells. Progr Immunol 1989;7:243-57.
60. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB. Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 1989;169:1277-94.
61. Vroom TM, Bos JD, Borst J. Scientific correspondence. Nature 1989;341:114.
62. Koning F, Maloy WL, Cohen D, Coligan JE. Independent association of T cell receptor β and γ chains with CD3 in the same cell. J Exp Med 1987;166:595-600.
63. De Waal Malefyt R, Alarcon B, Yssel H, Sancho J, Miyajima A, Terhorst CP, Spits H, De Vries JE. Introduction of T cell receptor (TcR)-α cDNA has differential effects on TcR-γδ/CD3 expression by peer and lyon-1 cells. J Immunol 1989;142:3634-42.
64. Saito T, Hochstenbach F, Marusic-Galesic S, Kruisbeek AM, Brenner M, Germain RN. Surface expression of only γδ and/or αβ T cell receptor heterodimers by cells with four (α, β, γ, δ) functional receptor chains. J Exp Med 1988;168:1003-20.
65. Hochstenbach F, Brenner MB. T-cell receptor δ-chain can substitute for α to form a βδ heterodimer. Nature 1989;340:562-5.

66. Hayday AC, Diamond DJ, Tanigawa G, Heilig JS, Folsom V, Saito H, Tonegawa S. Unusual organization and diversity of T-cell receptor α-chain genes. Nature 1985;316:828-32.
67. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.
68. Lindsten T, Fowlkes BJ, Samelson LE, Davis MM, Chien Y-H. Transient rearrangements of the T cell antigen receptor α locus in early thymocytes. J Exp Med 1987;166:761-5.
69. Griesser H, Champagne E, Tkachuk D, Takihara Y, Lalande M, Baillie E, Minden M, Mak TW. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. Eur J Immunol 1988;18:641-4.
70. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-8.
71. Champagne E, Sagman U, Biondi A, Lewis WH, Mak TW, Minden MD. Structure and rearrangement of the T cell receptor J alpha locus in T cells and leukemic T cell lines. Eur J Immunol 1988;18:1033-8.
72. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-7.
73. Sims JE, Tunnacliffe A, Smith WJ, Rabbitts TH. Complexity of human T-cell antigen receptor β-chain constant- and variable-region genes. Nature 1984;312:541-5.
74. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-6.
75. Tunnacliffe A, Rabbitts TH. Sequence of the Dβ2-Jβ2 region of the human T-cell receptor β-chain locus. Nucleic Acids Res 1985;13:6651-61.
76. Toyonaga B, Yorshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8.
77. Lefranc M-P, Rabbitts TH. Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types. Nature 1985;316:464-6.
78. Quertermous T, Murre C, Dialynas D, Duby AD, Strominger JL, Waldman TA, Seidman JG. Human T-cell γ chain genes: organization, diversity, and rearrangement. Science 1986;231:252-5.
79. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
80. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
81. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-50.
82. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc M-P, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-83.
83. Loh EY, Lanier LL, Turck CW, Littman DR, Davis MM, Chien Y-H, Weiss A. Identification and sequence of a fourth human T cell antigen receptor chain. Nature 1987;330:569-72.
84. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-94.
85. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-101.
86. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-70.
87. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. The human T-cell receptor delta chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
88. Tunnacliffe A, Kefford R, Milstein C, Forster A, Rabbitts TH. Sequence and evolution of the human T-cell antigen receptor β-chain genes. Proc Natl Acad Sci USA 1985;82:5068-72.
89. Lefranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of

the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-600.
90. Pelicci PG, Subar M, Weiss A, Dalla-Favera R, Littman DR. Molecular diversity of the human T-gamma constant region genes. Science 1987;237:1051-5.
91. Li Y, Szabo P, Posnett DN. Molecular genotypes of the human T cell receptor γ-chain. J Immunol 1988;140:1300-3.
92. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-8.
93. Krangel MS, Band H, Hata S, McLean J, Brenner MB. Structurally divergent human T cell receptor γ proteins encoded by distinct Cγ genes. Science 1987;237:64-7.
94. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-28.
95. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$, $CD8^-$ T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. J Exp Med 1987;165:1076-94.
96. Christmas SE. Most human $CD3^+WT31^-$ clones with T cell receptor Cγ1 rearrangements show strong non-MHC-restricted cytotoxic activity in contrast to those with Cγ2 rearrangements. Eur J Immunol 1989;19:741-6.
97. Grossi CE, Ciconne E, Migone N, Bottino C, Zarcone D, Mingari MC, Ferrini S, Tambussi G, Viale O, Casorati G, Millo R, Moretta L, Moretta A. Human T cells expressing the γ/δ T-cell receptor (TcR-1): Cγ1- and Cγ2-encoded forms of the receptor correlate with distinctive morphology, cytoskeletal organization, and growth characteristics. Proc Natl Acad Sci USA 1989;86:1619-23.
98. Ruddle FH, Kucherlapati RS. Hybrid cells and human genes. Sci Am 1974;231(7):36-44.
99. McKusick VA. The anatomy of the human genome. J Hered 1980;71:370-91.
100. Shows TB, Sakaguchi AY, Naylor SL. Mapping the human genome, cloned genes, DNA polymorphisms, and inherited disease. In: Harris H, Hirschkorn K, eds. Adv Hum Genet. New York: Plenum Press, 1982;12:-341-452.
101. Harper ME, Saunders GF. Localization of single-copy genes on human chromosomes by *in situ* hybridization of ^3H-probes and autoradiography. In: Sparkes RS, De la Cruz FF, eds. Research perspectives in cytogenetics. Baltimore: University Park Press, 1984:117-33.
102. Donlon TA. Practical approaches to *in situ* hybridization. Karyogram 1986;12:3-10.
103. Croce CM, Shander M, Martinis J, Cicurel L, D'Ancona GG, Dolby TW, Koprowski H. Chromosomal location of the genes for human immunoglobulin heavy chains. Proc Natl Acad Sci USA 1979;76:3416-9.
104. Kirch IR, Morton CC, Nakahara K, Leder P. Human immunoglobulin heavy chain genes map to a region of translocations in malignant B lymphocytes. Science 1982;216:301-3.
105. McBride OW, Hieter PA, Hollis GF, Swan D, Otey MC, Leder P. Chromosomal location of human kappa and lambda immunoglobulin light chain constant region genes. J Exp Med 1982;155:1480-90.
106. Malcolm S, Barton P, Murphy C, Ferguson-Smith MA, Bentley DL, Rabbitts TH. Localization of human immunoglobulin κ light chain variable region genes to the short arm of chromosome 2 by *in situ* hybridization. Proc Natl Acad Sci USA 1982;79:4957-61.
107. Erikson J, Martinis J, Croce CM. Assignment of the genes for human λ immunoglobulin chains to chromosome 22. Nature 1981;294:173-5.
108. De la Chapelle A, Lenoir G, Boué J, Boué A, Gallano P, Huerre C, Szajnert M-F, Jeanpierre M, Lalouel J-M, Kaplan J-C. Lambda Ig constant region genes are translocated to chromosome 8 in Burkitt's lymphoma with t(8;22). Nucleic Acids Res 1983;11:1133-42.
109. Collins MKL, Goodfellow PN, Spurr NK, Solomon E, Tanigawa G, Tonegawa S, Owen MJ. The human T-cell receptor α-chain gene maps to chromosome 14. Nature 1985;314:273-4.
110. Croce CM, Isobe M, Palumbo A, Puck J, Ming J, Tweardy D, Erikson J. Gene for α-chain of human T-cell receptor: location on chromosome 14 region involved in T-cell neoplasms. Science 1985;227:1044-7.
111. Caccia N, Bruns GAP, Kirsch IR, Hollis GF, Bertness V, Mak TW. T cell receptor α chain genes are located on chromosome 14 AT 14q11-14q12 in humans. J Exp Med 1985;161:1255-60.
112. Barker PE, Royer H-D, Ruddle FH, Reinherz EL. Regional location of T cell receptor gene Tiα on human

chromosome 14. J Exp Med 1985;162:387-92.
113. Rabbitts TH, Lefranc MP, Stinson MA, Sims JE, Schroder J, Steinmetz M, Spurr NL, Solomon E, Goodfellow PN. The chromosomal location of T-cell receptor genes and a T cell rearranging gene: possible correlation with specific translocations in human T cell leukaemia. EMBO J 1985;4:1461-5.
114. Collins MKL, Goodfellow PN, Dunne MJ, Spurr NK, Solomon E, Owen MJ. A human T-cell antigen receptor β chain gene maps to chromosome 7. EMBO J 1984;3:2347-9.
115. Morton CC, Duby AD, Eddy RL, Shows TB, Seidman JG. Genes for β chain of human T-cell antigen receptor map to regions of chromosomal rearrangement in T cells. Science 1985;228:582-4.
116. Le Beau MM, Diaz MO, Rowley JD, Mak TW. Chromosomal localization of the human T cell receptor β-chain genes. Cell 1985;41:335.
117. Isobe M, Erikson J, Emanuel BS, Nowell PC, Croce CM. Location of gene for β subunit of human T-cell receptor at band 7q35, a region prone to rearrangements in T cells. Science 1985;228:580-2.
118. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. Nature 1985;316:54952.
119. Erikson J, Finan J, Nowell PC, Croce CM. Translocation of immunoglobulin VH genes in Burkitt lymphoma. Proc Natl Acad Sci USA 1982;79:5611-5.
120. Erikson J, Nishikura K, ar-Rushdi A, Finan J, Emanuel B, Lenoir G, Nowell PC, Croce CM. Translocation of an immunoglobulin κ locus to a region 3' of an unrearranged c-*myc* oncogene enhances c-*myc* transcription. Proc Natl Acad Sci USA 1983;80:7581-5.
121. Croce CM, Thierfelder W, Erikson J, Nishikura K, Finan J, Lenoir GM, Nowell PC. Transcriptional activation of an unrearranged and untranslocated c-*myc* oncogene by translocation of a Cλ locus in Burkitt lymphoma cells. Proc Natl Acad Sci USA 1983;80:6922-6.
122. Erikson J, Williams DL, Finan J, Nowell PC, Croce CM. Locus of the α-chain of the T-cell receptor is split by chromosome translocation in T-cell leukemias. Science 1985;229:784-6.
123. Baer R, Chen K-C, Smith SD, Rabbitts TH. Fusion of an immunoglobulin variable gene and a T cell receptor constant gene in the chromosome 14 inversion associated with T cell tumors. Cell 1985;43:705-13.
124. Erikson J, Finger L, Sun L, Ar-Rushdi A, Nishikura K, Minowada J, Finan J, Emanuel BS, Nowell PC, Croce CM. Deregulation of c-*myc* by translocation of the α locus of the T-cell receptor in T-cell leukemias. Science 1986;232:884-6.
125. Kagan J, Finan J, Letofsky J, Besa EC, Nowell PC, Croce CM. α-Chain locus of the T-cell antigen receptor is involved in the t(10;14) chromosome translocation of T-cell acute lymphocytic leukemia. Proc Natl Acad Sci USA 1987;84:4543-6.
126. Reynolds TC, Smith SD, Sklar J. Analysis of DNA surrounding the breakpoints of chromosomal translocations involving the β T cell receptor gene in human lymphoblastic neoplasms. Cell 1987;50:107-17.
127. Cleary ML, Mellentin JD, Spies J, Smith SD. Chromosomal translocation involving the β T cell receptor gene in acute leukemia. J Exp Med 1988;167:682-7.
128. Yunis JJ, Oken MM, Kaplan ME, Ensrud KM, Howe RR, Theologides A. Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. N Engl J Med 1982;307:1231-6.
129. Bloomfield CD, Arthur DC, Frizzera G, Levine EG, Peterson BA, Gajl-Peczalska KJ. Nonrandom chromosome abnormalities in lymphoma. Cancer Res 1983;43:2975-84.
130. Williams DL, Look AT, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S. New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia. Cell 1984;36:101-9.
131. Showe LC, Croce CM. Chromosome translocations in B and T cell neoplasias. Semin Hematol 1986;23:237-44.
132. Aurias A, Dutrillaux B, Buriot D, Lejeune J. High frequencies of inversions and translocations of chromosomes 7 and 14 in ataxia telangiectasia. Mutat Res 1980;69:369-74.
133. O'Connor RD, Brown MG, Francke U. Immunologic and karyotypic studies in ataxia-telangiectasia; specificity of break points on chromosomes 7 and 14 in lymphocytes from patiens and relatives. In: Bridges BA, Harnden DG, eds. Ataxia-telangiectasia - a cellular and molecular link between cancer, neuropathology, and immune deficiency. Chichester: John Wiley and Sons, 1982:259-70.
134. Hecht F, Hecht BK. Ataxia-telangiectasia breakpoints in chromosome rearrangements reflect genes important

to T and B lymphocytes. In: Gatti RA, Swift M, eds. Ataxia-telangiectasia: genetics, neuropathology, and immunology of a degenerative disease of childhood. New York: Alan R. Liss, 1985:189-95.
135. Gatti RA, Aurias A, Griscelli C, Sparkes RS. Translocations involving chromosomes 2p and 22q in ataxia-telangiectasia. Disease markers 1985;3:169-75.
136. Aurias A, Dutrillaux B. Probable involvement of immunoglobulin superfamily genes in most recurrent chromosomal rearrangements from ataxia telangiectasia. Hum Genet 1986;72:210-4.
137. Butterworth SV, Taylor AMR. A subpopulation of t(2;14)(p11;q32) cells in ataxia telangiectasia B lymphocytes. Hum Genet 1986;73:346-9.
138. Welch JP, Lee CLY, Beatty-DeSana JW, Hoggard MJ, Cooledge JW, Hecht F, Kaiser McCaw B, Robinson A. Non-random occurrence of 7-14 translocations in human lymphocyte cultures. Nature 1975;255:241-4.
139. Aymé S, Mattei JF, Mattei MG, Aurran Y, Giraud F. Nonrandom distribution of chromosome breaks in cultured lymphocytes of normal subjects. Hum Genet 1976;31:161-75.
140. Zech L, Haglund U. A recurrent structural aberration, t(7;14), in phytohemagglutinin-stimulated lymphocytes. Hereditas 1978;89:69-73.
141. Mattei MG, Ayme S, Mattei JF, Aurran Y, Giraud F. Distribution of spontaneous chromosome breaks in man. Cytogenet Cell Genet 1979;23:95-102.
142. Wallace C, Bernstein R, Pinto MR. Non-random in vitro 7;14 translocations detected in a routine cytogenetic series: 12 examples and their possible significance. Hum Genet 1984;66:157-61.
143. Aurias A, Couturier J, Dutrillaux A-M, Dutrillaux B, Herpin F, Lamolaitte E, Lombard M, Muleris M, Paravatou M, Prieur M, Prod'homme M, Sportes M, Viegas-Péquignot E, Volobouev V. Inversion (14)(q12qter) or (q11.2q32.3): the most frequently acquired rearrangement in lymphocytes. Hum Genet 1985;71:19-21.
144. Boder E. Ataxia-telangiectasia: an overview. In: Gatti RA, Swift M, eds. Ataxia-telangiectasia: genetics, neuropathology, and immunology of a degenerative disease of childhood. New York: Alan R. Liss, 1985:1-63.
145. Dalla-Favera R, Bregni M, Erikson J, Patterson D, Gallo RC, Croce CM. Human c-*myc onc* gene is located on the region of chromosome 8 that is translocated in Burkitt lymphoma cells. Proc Natl Acad Sci USA 1982;79:7824-7.
146. Bernheim A, Berger R, Lenoir G. Cytogenetic studies on African Burkitt's lymphoma cell lines: t(8;14), t(2;8) and t(8;22) translocations. Cancer Genet Cytogenet 1981;3:307-15.
147. Croce CM, Nowell PC. Molecular basis of human B cell neoplasia. Blood 1985;65:1-7.
148. Waldmann TA, Korsmeyer SJ, Bakshi A, Arnold A, Kirsch IR. Molecular genetic analysis of human lymphoid neoplasms: immunoglobulin genes and the c-*myc* oncogene. Ann Intern Med 1985;102:497-510.
149. Alt FW, Blackwell TK, Yancopoulos GD. Development of the primary antibody repertoire. Science 1987;238:1079-87.
150. Wu TT, Kabat EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. J Exp Med 1970;132:211-50.
151. Kabat EA. Antibody diversity versus antibody complementarity. Pharmacol Rev 1982;34:23-38.
152. Kabat EA. Antibody complementarity and antibody structure. J Immunol 1988;S25-S36.
153. Davies DR, Metzger H. Structural basis of antibody function. Annu Rev Immunol 1983;1:87-117.
154. Amit AG, Mariuzza RA, Phillips SEV, Poljak RJ. Three-dimensional structure of an antigen-antibody complex at 2.8 Å resolution. Science 1986;233:747-53.
155. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402 and corrigenda 1988;335:744.
156. Novotny J, Tonegawa S, Saito H, Kranz DM, Eisen HN. Secondary, tertiary, and quaternary structure of T-cell-specific immunoglobulin-like polypeptide chains. Proc Natl Acad Sci USA 1986;83:742-6.
157. Claverie J-M, Prochnicka-Chalufour A, Bougueleret L. Implications of a Fab-like structure for the T-cell receptor. Immunol Today 1989;10:10-4.
158. Berek C, Milstein C. Mutation drift and repertoire shift in the maturation of the immune response. Immunol Rev 1987;96:23-41.
159. Rajewsky K, Förster I, Cumano A. Evolutionary and somatic selection of the antibody repertoire in the mouse. Science 1987;238:1088-94.
160. Early P, Huang H, Davis M, Calame K, Hood L. An immunoglobulin heavy chain variable region gene is generated from three segments of DNA: VH, D and JH. Cell 1980;19:981-92.

161. Sakano H, Kurosawa Y, Weigert M, Tonegawa S. Identification and nucleotide sequence of a diversity DNA segment (D) of immunoglobulin heavy-chain genes. Nature 1981;290:562-5.
162. Fujimoto S, Yamagishi H. Isolation of an excision product of T-cell receptor α-chain gene rearrangements. Nature 1987;327:242-3.
163. Okazaki K, Davis DD, Sakano H. T cell receptor β gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. Cell 1987;49:477-85.
164. Okazaki K, Sakano H. Thymocyte circular DNA excised from T cell receptor α-δ gene complex. EMBO J 1988;7:1669-74.
165. Winoto A, Baltimore D. Separate lineages of T cells expressing the αβ and γδ receptors. Nature 1989;338:430-2.
166. Reth M, Leclercq L. Assembly of immunoglobulin variable region gene segments. In: Calabi F, Neuberger MS, eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier Science Publishers 1987:111-34.
167. Akira S, Okazaki K, Sakano H. Two pairs of recombination signals are sufficient to cause immunoglobulin V-(D)-J joining. Science 1987;238:1134-8.
168. Blackwell TK, Alt FW. Site-specific recombination between immunoglobulin D and JH segments that were introduced into the genome of a murine pre-B cell line. Cell 1984;37:105-12.
169. Yancopoulos GD, Blackwell TK, Suh H, Hood L, Alt FW. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell 1986;44:251-9.
170. Desiderio SV, Wolff KR. Rearrangement of exogenous immunoglobulin VH and DJH gene segments after retroviral transduction into immature lymphoid cell lines. J Exp Med 1988;167:372-89.
171. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-22.
172. Kurosawa Y, Tonegawa S. Organization, structure, and assembly of immunoglobulin heavy chain diversity DNA segments. J Exp Med 1982;155:201-18.
173. Malissen M, McCoy C, Blanc D, Trucy J, Devaux C, Schmitt-Verhulst A-M, Fitch F, Hood L, Malissen B. Direct evidence for chromosomal inversion during T-cell receptor β-gene rearrangements. Nature 1986;319:28-33.
174. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.
175. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
176. Lewis S, Rosenberg N, Alt F, Baltimore D. Continuing kappa-gene rearrangement in a cell line transformed by Abelson murine leukemia virus. Cell 1982;30:807-16.
177. Feddersen RM, Van Ness BG. Double recombination of a single immunoglobulin κ-chain allele: implications for the mechanism of rearrangement. Proc Natl Acad Sci USA 1985;82:4793-7.
178. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
179. Foa R, Migone N, Fierro MT, Basso G, Lusso P, Putti MC, Giubellino MC, Saitta M, Miniero R, Casorati G, Gavosto F. Genotypic characterization of common acute lymphoblastic leukemia may improve the phenotypic classification. Exp Hematol 1987;15:942-5.
180. Boehm TLJ, Werle A, Ganser A, Kornhuber B, Drahovsky D. T cell receptor γ chain variable gene rearrangements in acute lymphoblastic leukemias of T and B lineage. Eur J Immunol 1987;17:1593-7.
181. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
182. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
183. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Comparison of T cell receptor α, β,

and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J Clin Invest 1988;81:989-96.

184. Reth MG, Jackson S, Alt FW. VHDJH formation and DJH replacement during pre-B differentiation: non-random usage of gene segments. EMBO J 1986;5:2131-8.
185. Reth M, Gehrmann P, Petrac E, Wiese P. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. Nature 1986;322:840-2.
186. Kleinfield R, Hardy RR, Tarlinton D, Dangl J, Herzenberg LA, Weigert M. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1^+ B-cell lymphoma. Nature 1986;322:843-6.
187. Marolleau J-P, Fondell JD, Malissen M, Trucy J, Barbier E, Marcy KB, Cazeenave P-A, Primi D. The joining of germ-line Vα to Jα genes replaces the preexisting Vα-Jα complexes in a T cell receptor α,β positive T cell line. Cell 1988;55:291-300.
188. Toda M, Fujimoto S, Iwasato T, Takeshita S, Tezuka K, Ohbayashi T, Yamagishi H. Structure of extrachromosomal circular DNAs excised from T-cell antigen receptor alpha and delta-chain loci. J Mol Biol 1988;202:219-31.
189. Fondell JD, Marolleau J-P, Primi D, Marcu KB. On the mechanism of non-allelically excluded Vα-Jα T cell receptor secondary rearrangements in a murine T cell lymphoma. J Immunol 1990;144:1094-103.
190. Epplen JT, Bartels F, Becker A, Nerz G, Prester M, Rinaldy A, Simon MM. Change in antigen specificity of cytotoxic T lymphocytes is associated with the rearrangement and expression of a T-cell receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4441-5.
191. Epplen JT, Chluba J, Hardt C, Hinkkanen A, Steimle V, Stockinger H. Mammalian T-lymphocyte antigen receptor genes: genetic and nongenetic potential to generate variability. Hum Genet 1987;75:300-10.
192. Kodaira M, Kinashi T, Umemura I, Matsuda F, Noma T, Ono Y, Honjo T. Organization and evolution of variable region genes of the human immunoglobulin heavy chain. J Mol Biol 1986;190:529-41.
193. Lee KH, Matsuda F, Kinashi T, Kodaira M, Honjo T. A novel family of variable region genes of the human immunoglobulin heavy chains. J Mol Biol 1987;195:761-8.
194. Schroeder HW, Hillson JL, Perlmutter RM. Early restriction of the human antibody repertoire. Science 1987;238:791-3.
195. Humphries CG, Shen A, Kuziel WA, Capra JD, Blattner FR, Tucker PW. A new human immunoglobulin VH family preferentially rearranged in immature B-cell tumours. Nature 1988;331:446-9.
196. Pech M, Smola H, Pohlenz H-D, Straubinger B, Gerl R, Zachau HG. A large section of the gene locus encoding human immunoglobulin variable regions of the kappa type is duplicated. J Mol Biol 1985;183:291-9.
197. Klobeck H-G, Bornkamm GW, Combriato G, Mocikat R, Pohlenz H-D, Zachau HG. Subgroup IV of human immunoglobulin κ light chains is encoded by a single germline gene. Nucleic Acids Res 1985;13:6515-29.
198. Brodeur PH. Genes encoding the immunoglobulin variable regions. In: Calibi F, Neuberger MS, eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier Science Publishers, 1987:81-109.
199. Anderson MLM, Szajnert MF, Kaplan JC, McColl L, Young BD. The isolation of a human Ig Vλ gene from a recombinant library of chromosome 22 and estimation of its copy number. Nucleic Acids Res 1984;12:6647-61.
200. Anderson MLM, Brown L, McKenzie E, Kellow JE, Young BD. Cloning and sequence analysis of an Igλ light chain mRNA expressed in the Burkitt's lymphoma cell line EB4. Nucleic Acids Res 1985;13:2931-41.
201. Yoshikai Y, Kimura N, Toyonaga B, Mak TW. Sequences and repertoire of human T cell receptor α chain variable region genes in mature T lymphocytes. J Exp Med 1986;164:90-103.
202. Klein MH, Concannon P, Everett M, Kim LDH, Hunkapiller T, Hood L. Diversity and structure of human T-cell receptor α-chain variable region genes. Proc Natl Acad Sci USA 1987;84:6884-8.
203. Kimura N, Toyonaga B, Yoshikai Y, Du R-P, Mak TW. Sequences and repertoire of the human T cell receptor α and β chain variable region genes in thymocytes. Eur J Immunol 1987;17:375-83.
204. Toyonaga B, Mak TW. Genes of the T-cell antigen receptor in normal and malignant T cells. Annu Rev Immunol 1987;5:585-620.
205. Kimura N, Toyonaga B, Yoshikai Y, Triebel F, Debre P, Minden MD, Mak TW. Sequences and diversity of human T cell receptor β chain variable region genes. J Exp Med 1986;164:739-50.
206. Concannon P, Pickering LA, Kung P, Hood L. Diversity and structure of human T-cell receptor β-chain

variable region genes. Proc Natl Acad Sci USA 1986;83:6598-602.
207. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
208. LeFranc M-P, Rabbits TH. The human T-cell receptor γ (TRG) genes. Trends Biochem Sciences 1989;14:214-8.
209. Guglielmi P, Davi F, d'Auriol L, Bories J-C, Dausset J, Bensussan A. Use of a variable α region to create a functional T-cell receptor δ chain. Proc Natl Acad Sci USA 1988;85:5634-8.
210. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-7.
211. Elliott JF, Rock EP, Patten PA, Davis MM, Chien Y. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. Nature 1988;331:627-31.
212. Hata S, Brenner MB, Krangel MS. Identification of putative human T cell receptor δ complementary DNA clones. Science 1987;238:678-82.
213 Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-8.
214. Yancopoulos GD, Desiderio SV, Paskind M, Kearney JF, Baltimore D, Alt FW. Preferential utilization of the most JH-proximal VH gene segments in pre-B-cell lines. Nature 1984;311:727-33.
215. Mizutani S, Ford AM, Wiedemann LM, Chan LC, Furley AJW, Greaves MF, Molgaard HV. Rearrangement of immunoglobulin heavy chain genes in human T leukaemic cells shows preferential utilization of the D segment (DQ_{52}) nearest to the J region. EMBO J 1986;5:3467-73.
216. Leiden JM, Dialynas DP, Duby AD, Murre C, Seidman J, Strominger JL. Rearrangement and expression of T-cell antigen receptor genes in human T-lymphocyte tumor lines and normal human T-cell clones: evidence for allelic exclusion of Tiβ gene expression and preferential use of a Jβ2 gene segment. Mol Cell Biol 1986;6:3207-14.
217. Jitsukawa S, Faure F, Lipinski M, Triebel F, Hercend T. A novel subset of human lymphocytes with a T cell receptor-γ complex. J Exp Med 1987;166:1192-7.
218. Bottino C, Tambussi G, Ferrini S, Ciccone E, Varese P, Mingari MC, Moretta L, Moretta A. Two subsets of human T lymphocytes expressing γ/δ antigen receptor are identifiable by monoclonal antibodies directed to two distinct molecular forms of the receptor. J Exp Med 1988;168:491-505.
219. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-8.
220. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.
221. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-5.
222. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
223. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
224. Landau NR, Schatz DG, Rosa M, Baltimore D. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol Cell Biol 1987;7:3237-43.
225. Siu G, Kronenberg M, Strauss E, Haars R, Mak TW, Hood L. The structure, rearrangement and expression of Dβ gene segments of the murine T-cell antigen receptor. Nature 1984;311:344-50.
226. Barth RK, Kim BS, Lan NC, Hunkapiller T, Sobieck N, Winoto A, Gershenfeld H, Okada C, Hansburg D, Weissman IL, Hood L. The murine T-cell receptor uses a limited repertoire of expressed Vβ gene segments. Nature 1985;316:517-23.
227. Behlke MA, Spinella DG, Chou HS, Sha W, Hartl DL, Loh DY. T-cell receptor β-chain expression: dependence on relatively few variable region genes. Science 1985;229:566-70.
228. Heilig JS, Glimcher LH, Kranz DM, Clayton LK, Greenstein JL, Saito H, Maxam AM, Burakoff SJ, Eisen HN,

Tonegawa S. Expression of the T-cell specific γ gene is unnecessary in T cells recognizing class II MHC determinants. Nature 1985;317:68-70.
229. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-7.
230. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-4.
231. Alt F, Rosenberg N, Lewis S, Thomas E, Baltimore D. Organization and reorganization of immunoglobulin genes in A-MulV-transformed cells: rearrangement of heavy but not light chain genes. Cell 1981;27:381-90.
232. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, Coffman R, Rosenberg N, Tonegawa S, Baltimore D. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J 1984;3:1209-19.
233. Korsmeyer SJ, Hieter PA, Ravetch JV, Poplack DG, Waldmann TA, Leder P. Developmental hierarchy of immunoglobulin gene rearrangements in human leukemic pre-B-cells. Proc Natl Acad Sci USA 1981;78:7096-100.
234. Nadler LM, Korsmeyer SJ, Anderson KC, Boyd AW, Slaughenhoupt B, Park E, Jensen J, Coral F, Mayer RJ, Sallan SE, Ritz J, Schlossman SF. B cell origin of non-T cell acute lymphoblastic leukemia: a model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Invest 1984;74:332-40.
235. Ha K, Hozumi N, Hrincu A, Gelfand EW. Lineage specific classification of leukaemia: results of the analysis of sixty cases of childhood leukaemia. Br J Haematol 1985;61:237-49.
236. Foa R, Migone N, Basso G, Cattoretti G, Pizzolo G, Lauria F, Casorati G, Giubellino MC, Capuzzo F, Cantù-Rajnoldi A, Lusso P, Carbonara AO, Gavosto F. Molecular and immunological evidence of B-cell commitment in "null" acute lymphoblastic leukaemia. Int J Cancer 1986;38:317-23.
237. Felix CA, Reaman GH, Korsmeyer SJ, Hollis GF, Dinndorf PA, Wright JJ, Kirsch IR. Immunoglobulin and T cell receptor gene configuration in acute lymphoblastic leukemia of infancy. Blood 1987;70:536-41.
238. Hieter PA, Korsmeyer SJ, Waldmann TA, Leder P. Human immunoglobulin κ light-chain genes are deleted or rearranged in λ-producing B cells. Nature 1981;290:368-72.
239. Korsmeyer SJ, Greene WC, Cossman J, Hsu S-M, Jensen JP, Neckers LM, Marshall SL, Bakhshi A, Depper JM, Leonard WJ, Jaffe ES, Waldmann TA. Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia. Proc Natl Acad Sci USA 1983;80:4522-6.
240. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE Jr, Whitcomb CC, Thomas IV CY. Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 1987;69:79-86.
241. Foroni L, Catovsky D, Luzzatto L. Immunoglobulin gene rearrangements in hairy cell leukemia and other chronic B cell lymphoproliferative disorders. Leukemia 1987;1:389-92.
242. Korsmeyer SJ, Hieter PA, Sharrow SO, Goldman CK, Leder P, Waldmann TA. Normal human B cells display ordered light chain gene rearrangements and deletions. J Exp Med 1982;156:975-85.
243. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of κ genes in human B cells. Nature 1985;316:260-2.
244. Graninger WB, Goldman PL, Morton CC, O'Brien SJ, Korsmeyer SJ. The κ-deleting element: germline and rearranged, duplicated and dispersed forms. J Exp Med 1988;167:488-501.
245. Klobeck H-G, Zachau HG. The human Cκ gene segment and the kappa deleting element are closely linked. Nucleic Acids Res 1986;14:4591-603.
246. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.
247. Chien Y, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature 1987;327:677-82.
248. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-7.
249. Born W, Yagüe J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor β-chain genes during T-cell development. Proc Natl Acad Sci USA 1985;82:2925-9.
250. Born W, Rathbun G, Tucker P, Marrack P, Kappler J. Synchronized rearrangement of T-cell γ and β chain genes in fetal thymocyte development. Science 1986;234:479-82.

251. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.
252. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
253. Pardoll DM, Fowlkes BJ, Bluestone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
254. Bluestone JA, Pardoll D, Sharrow SO, Fowlkes BJ. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. Nature 1987;326:82-4.
255. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 1988;335:443-5.
256. Greenberg JM, Quertermous T, Seidman JG, Kersey JH. Human T cell γ-chain gene rearrangements in acute lymphoid and nonlymphoid leukemia: comparison with the T cell receptor β-chain gene. J Immunol 1986;137:2043-9.
257. Davey MP, Bongiovanni KF, Kaulfersch W, Quertermous T, Seidman JG, Hershfield MS, Kurtzberg J, Haynes BF, Davis MM, Waldmann TA. Immunoglobulin and T-cell receptor gene rearrangement and expression in human lymphoid leukemia cells at different stages of maturation. Proc Natl Acad Sci USA 1986;83:8759-63.
258. Goorha R, Bunin N, Mirro J Jr, Murphy SB, Cross AH, Behm FG, Quertermous T, Seidman J, Kitchingman GR. Provocative pattern of rearrangements of the genes for the γ and β chains of the T-cell receptor in human leukemias. Proc Natl Acad Sci USA 1987;84:4547-51.
259. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ. T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
260. Le Paslier D, Chen Z, Loiseau P, Cohen D, Sigaux F. T cell rearranging gene γ: diversity and mRNA expression in fresh cells from T cell acute lymphoblastic leukemia. Blood 1987;70:637-46.
261. Foroni L, Foldi J, Matutes E, Catovsky D, O'Connor NJ, Baer R, Forster A, Rabbitts TH, Luzzatto L. α, β and γ T-cell receptor genes: rearrangements correlate with haematological phenotype in T cell leukaemias. Br J Haematol 1987;67:307-18.
262. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-82.
263. Foroni L, Laffan M, Boehm T, Rabbitts TH, Catovsky D, Luzzatto L. Rearrangement of the T-cell receptor δ genes in human T-cell leukemias. Blood 1989;73:559-65.
264. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334-42.
265. Matsuoka M, Asou N, Hattori T, Matsuo T, Nishimura H, Takatsuki K. T cell γ chain gene rearrangement without T cell receptor β chain gene rearrangement in two cases of non-Hodgkin's lymphoma. Acta Haematol 1987;77:172-6.
266. Weiss LM, Picker LJ, Grogan TM, Warnke RA, Sklar J. Absence of clonal beta and gamma T-cell receptor gene rearrangements in a subset of peripheral T-cell lymphomas. Am J Pathol 1988;130:436-42.
267. Triebel F, Lefranc M-P, Hercend T. Further evidence for a sequentially ordered activation of T cell rearranging gamma genes during T lymphocyte differentiation. Eur J Immunol 1988;18:789-94.
268. Subar M, Pelicci PG, Neri A, Allavena P, Littman DR, Knowles II DM, Dalla-Favera R. Patterns of T cell receptor gamma gene rearrangement and expression in B and T lymphoid malignancies. Leukemia 1988;2:19-26.
269. Tawa A, Benedict SH, Hara J, Hozumi N, Gelfand EW. Rearrangement of the T cell receptor γ-chain gene in childhood acute lymphoblastic leukemia. Blood 1987;70:1933-9.
270. De Villartay J-P, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-12.
271. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-4.
272. Hockett RD Jr, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-74.
273. Winoto A, Baltimore D. Separate lineages of T cells expressing the $\alpha\beta$ and $\gamma\delta$ receptors. Nature 1989;338:430-2.

274. Yoshikai Y, Reis MD, Mak TW. Athymic mice express a high level of functional γ-chain but greatly reduced levels of α- and β-chain T-cell receptor messages. Nature 1986;324:482-5.
275. Yoshikai Y, Matsuzaki G, Takeda Y, Ohga S, Kishihara K, Yuuki H, Nomota K. Functional T cell receptor δ chain gene messages in athymic nude mice. Eur J Immunol 1988;18:1039-43.
276. Van Dongen JJM, Comans-Bitter WM, Friedrich W, Neyens HJ, Belohradsky BH, Kohn Th, Hagemeyer A, Borst J. Analysis of patients with DiGeorge anomaly provides evidence for extrathymic development of TcR-γδ$^+$ lymphocytes in man. Submitted for publication.
277. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 1983;131:1789-96.
278. Phillips JH, Lanier LL. Dissection of the lymphokine-activated killer phenomenon: relative contribution of peripheral blood natural killer cells and T lymphocytes to cytolysis. J Exp Med 1986;164:814-25.
279. Lanier LL, Phillips JH, Hackett J Jr, Tutt M, Kumar V. Natural killer cells: definition of a cell type rather than a function. J Immunol 1986;137:2735-9.
280. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986;136:4480-6.
281. Ritz J, Campen TJ, Schmidt RE, Royer HD, Hercend T, Hussey RE, Reinherz EL. Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. Science 1985;228:1540-3.
282. Lanier LL, Cwirla S, Federspiel N, Phillips JH. Human natural killer cells isolated from peripheral blood do not rearrange T cell antigen receptor β chain genes. J Exp Med 1986;163:209-14.
283. Lanier LL, Cwirla S, Phillips JH. Genomic organization of T cell γ genes in human peripheral blood natural killer cells. J Immunol 1986;137:3375-7.
284. Triebel F, Graziani M, Faure F, Jitsukawa S, Hercend T. Cloned human CD3$^-$ lymphocytes with natural killer-like activity do not express nor rearrange T cell receptor gamma genes. Eur J Immunol 1987;17:1209-12.
285. Pelicci P-G, Allavena P, Subar M, Rambaldi A, Pirelli A, Di Bello M, Barbui T, Knowles II DM, Dalla-Favera R, Mantovani A. T cell receptor (α, β, γ) gene rearrangements and expression in normal and leukemic large granular lymphocytes/natural killer cells. Blood 1987;70:1500-8.
286. Biondi A, Allavena P, Rossi V, Bassan R, Barbui T, Champagne E, Mak TW, Minden MD, Rambaldi A, Mantovani A. T cell receptor δ gene organization and expression in normal and leukemic natural killer cells. J Immunol 1989;143:1009-14.
287. Gathings WE, Lawton AR, Cooper MD. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur J Immunol 1977;7:804-10.
288. Link MP, Stewart SJ, Warnke RA, Levy R. Discordance between surface and cytoplasmic expression of the Leu-4 (T3) antigen in thymocytes and in blast cells from childhood T lymphoblastic malignancies. J Clin Invest 1985;76:248-53.
289. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
290. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
291. Swerdlow SH, Angermeier PA, Hartman AL. Intrathymic ontogeny of the T cell receptor associated CD3 (T3) antigen. Lab Invest 1988;58:421-7.
292. Mirro J Jr, Kitchingman G, Behm FG, Murphy SB, Goorha RM. T cell differentiation stages identified by molecular and immunologic analysis of the T cell receptor complex in childhood lymphoblastic leukemia. Blood 1987;69:908-12.
293. Pittaluga S, Uppenkamp M, Cossman J. Development of T3/T cell receptor gene expression in human pre-T neoplasms. Blood 1987;69:1062-7.
294. Janossy G, Coustan-Smith E, Campana D. The reliability of cytoplasmic CD3 and CD22 antigen expression in the immunodiagnosis of acute leukemia: a study of 500 cases. Leukemia 1989;3:170-81.
295. Brenner MB, Mclean J, Scheft H, Warnke RA, Jones N, Strominger JL. Characterization and expression of the human αβ T cell receptor by using a framework monoclonal antibody. J Immunol 1987;138:1502-9.
296. Picker LJ, Brenner MB, Weiss LM, Smith SD, Warnke RA. Discordant expression of CD3 and T-cell receptor

beta-chain antigens in T-lineage lymphomas. Am J Pathol 1987;129:434-40.
297. Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian W-T, Ip S, Wong L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. J Immunol 1989;142:57-66.
298. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
299. Tidman N, Janossy G, Bodger M, Granger S, Kung PC, Goldstein G. Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. Clin Exp Immunol 1981;45:457-67.
300. Michiels JJ, Adriaansen HJ, Hagemeijer A, Hooijkaas H, Van Dongen JJM, Abels J. TdT positive B-cell acute lymphoblastic leukaemia (B-ALL) without Burkitt characteristics. Br J Haematol 1988;68:423-6.
301. Drexler HG, Messmore HL, Menon M, Minowada J. A case of TdT-positive B-cell acute lymphoblastic leukemia. Am J Clin Pathol 1986;85:735-8.
302. Secker-Walker L, Stewart E, Norton J, Campana D, Thomas A, Hoffbrand V, Prentice G. Multiple chromosome abnormalities in a drug resistant TdT positive B-cell leukemia. Leuk Res 1987;11:155-61.
303. Calabi F. The immunoglobulin superfamily. In: Calabi F, Neuberger MS eds. Molecular genetics of immunoglobulin. Amsterdam: Elsevier Science Publishers 1987:203-39.
304. Williams AF. A year in the life of the immunoglobulin superfamily. Immunol Today 1987;8:298-303.
305. Williams AF. The immunoglobulin superfamily takes shape. Nature 1984; 308:12-3.
306. Hood L, Kronenberg M, Hunkapiller T. T cell antigen receptors and the immunoglobulin supergene family. Cell 1985;40:225-9.
307. Hunkapiller T, Hood L. The growing immunoglobulin gene superfamily. Nature 1986;323:15-6.
308. Williams AF, Barclay AN. The immunoglobulin superfamily. In: Honjo T, Alt FW, Rabbitts TH, eds. Immunoglobulin genes. London: Academic Press, 1989:361-87.
309. Reinherz EL, Meuer SC, Schlossman SF. The human T cell receptor: analysis with cytotoxic T cell clones. Immunol Rev 1983;74:83-112.
310. Swain SL. T cell subsets and the recognition of MHC class. Immunol Rev 1983;74:129-42.
311. Goverman J, Hunkapiller T, Hood L. A speculative view of the multicomponent nature of T cell antigen recognition. Cell 1986;45:475-84.
312. Marrack P, Kappler J. The antigen-specific, major histocompatibility complex-restricted receptor on T cells. Adv Immunol 1986;38:1-30.
313. Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of $\gamma\delta$ receptor-bearing T lymphocytes. Nature 1987;330:262-4.
314. Strominger JL. The $\gamma\delta$ T cell receptor and class Ib MHC-related proteins: enigmatic molecules of immune recognition. Cell 1989;57:895-8.
315. Bonneville M, Ito K, Krecko EG, Itohara S, Kappes D, Ishida I, Kanagawa O, Janeway CA Jr, Murphy DB, Tonegawa S. Recognition of a self major histocompatibility complex TL region product by $\gamma\delta$ T-cell receptors. Proc Natl Acad Sci USA 1989;86:5928-32.
316. Marrack P, Kappler J. The T cell and its receptor. Sci Am 1986;254(2):36-45.
317. Littman DR. The structure of the CD4 and CD8 genes. Annu Rev Immunol 1987;5:561-84.
318. Spits H, Borst J, Terhorst C, De Vries JE. The role of T cell differentiation markers in antigen-specific and lectin-dependent cellular cytotoxicity mediated by $T8^+$ and $T4^+$ human cytotoxic T cell clones directed at class I and class II MHC antigens. J Immunol 1982;129:1563-9.
319. Rotteveel FTM, Kokkelink I, Van Lier RAW, Kuenen B, Meager A, Miedema F, Lucas CJ. Clonal analysis of functionally distrinct human $CD4^+$ T cell subsets. J Exp Med 1988;168:1659-73.
320. Townsend ARM, Rothbard J, Gotch FM, Bahadur G, Wraith D, McMichael AJ. The epitopes of influenza nucleoprotein recognized by cytotoxic T lymphocytes can be defined with short synthetic peptides. Cell 1986;44:959-68.
321. Buus S, Colon S, Smith C, Freed JH, Miles C, Grey HM. Interaction between a "processed" ovalbumin peptide and Ia molecules. Proc Natl Acad Sci USA 1986;83:3968-71.
322. Guillet J-G, Lai M-Z, Briner TJ, Smith JA, Gefter ML. Interaction of peptide antigens and class II major histocompatibility complex antigens. Nature 1986;324:260-2.
323. Bjorkman PJ, Saper MA, Samraoui B, Bennett WS, Strominger JL, Wiley DC. Structure of the human class

I histocompatibility antigen, HLA-A2. Nature 1987;329:506-12.
324. Bjorkman PJ, Saper MA, Sarmraoui B, Bennett WS, Strominger JL, Wiley DC. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature 1987;329:512-8.
325. Brown JH, Jardetzky T, Saper MA, Samraoui B, Bjorkman PJ, Wiley DC. A hypothetical model of the foreign antigen binding site of class II histocompatibility molecules. Nature 1988;332:845-50.
326. Fink PJ, Matis LA, McElligott DL, Bookman M, Hedrick SM. Correlations between T-cell specificity and the structure of the antigen receptor. Nature 1986;321:219-26.
327. Kappler JW, Wade T, White J, Kushnir E, Blackman M, Bill J, Roehm N, Marrack P. A T cell receptor Vβ segment that imparts reactivity to a class II major histocompatibility complex product. Cell 1987;49:263-71.
328. Morel PA, Livingstone AM, Fathman CG. Correlation of T cell receptor Vβ gene family with MHC restriction. J Exp Med 1987;166:583-8.
329. Tan K-N, Datlof BM, Gilmore JA, Kronman AC, Lee JH, Maxam AM, Rao A. The T cell receptor Vα3 gene segment is associated with reactivity to p-Azobenzenearsonate. Cell 1988;54:247-61.
330. Baumhüter S, Fasel N, Corradin G, Guardiola J. T cell receptor β chain gene rearrangements and Vβ gene usage in horse cytochrome *c*-specific T cell hybridomas. Eur J Immunol 1987;17:657-61.
331. Schilham MW, Lang R, Acha-Orbea H, Benner R, Joho R, Hengartner H. Fine specificity and T-cell receptor β-chain gene rearrangements of five H-2D^b-specific cytotoxic T-cell clones. Immunogenetics:1987;25:171-8.
332. Engel I, Hedrick SM. Site-directed mutations in the VDJ junctional region of a T cell receptor β chain cause changes in antigenic peptide recognition. Cell 1988;54:473-84.
333. Swain SL. Significance of Lyt phenotypes: Lyt2 antibodies block activities of T cells that recognize class I major histocompatibility complex antigens regardless of their function. Proc Natl Acad Sci USA 1981;78:7101-5.
334. Marrack P, Endres R, Shimonkevitz R, Zlotnik A, Dialynas D, Fitch F, Kappler J. The major histocompatibility complex-restricted antigen receptor on T cells: II. role of the L3T4 product. J Exp Med 1983;158:1077-91.
335. Parnes JR. Molecular biology and function of CD4 and CD8. Adv Immunol 1989;44:265-311.
336. Emmrich F, Strittmatter U, Eichmann K. Synergism in the activation of human CD8 T cells by cross-linking the T-cell receptor complex with the CD8 differentiation antigen. Proc Natl Acad Sci USA 1986;83:8298-302.
337. Dembić Z, Haas W, Zamoyska R, Parnes J, Steinmetz M, Von Boehmer H. Transfection of the CD8 gene enhances T-cell recognition. Nature 1987;326:510-1.
338. Gay D, Maddon P, Sekaly R, Talle MA, Godfrey M, Long E, Goldstein G, Chess L, Axel R, Kappler J, Marrack P. Functional interaction between human T-cell protein CD4 and the major histocompatability complex HLA-DR antigen. Nature 1987;328:626-9.
339. Gabert J, Langlet C, Zamoyska R, Parnes JR, Schmitt-Verhulst A-M, Malissen B. Reconstitution of MHC class I specificity by transfer of the T cell receptor and Lyt-2 genes. Cell 1987;50:545-54.
340. Blue M-L, Craig KA, Anderson P, Branton KR Jr, Schlossman SF. Evidence for specific association between class I major histocompatibility antigens and the CD8 molecules of human suppressor/cytotoxic cells. Cell 1988;54:413-21.
341. Gay D, Buus S, Pasternak J, Kappler J, Marrack P. The T-cell accessory molecule CD4 recognizes a monomorphic determinant on isolated Ia. Proc Natl Acad USA 1988;85:5629-33.
342. Norment AM, Salter RD, Parham P, Engelhard VH, Littman DR. Cell-cell adhesion mediated by CD8 and MHC class I molecules. Nature 1988;336:79-81.
343. Rosenstein Y, Ratnofsky S, Burakoff SJ, Herrmann SH. Direct evidence for binding of CD8 to HLA class I antigens. J Exp Med 1989;169:149-60.
344. Saizawa K, Rojo J, Janeway CA Jr. Evidence for a physical association of CD4 and the CD3: α:β T-cell receptor. Nature 1987;328:260-3.
345. Takada S, Engleman EG. Evidence for an association between CD8 molecules and the T cell receptor complex on cytotoxic T cells. J Immunol 1987;139:3231-5.
346. Anderson P, Morimoto C, Breitmeyer JB, Schlossman SF. Regulatory interactions between members of the immunoglobulin superfamily. Immunol Today 1988;9:199-203.
347. Eisen HN, Siskind GW. Variations in affinities of antibodies during the immune response. Biochemistry 1964;3:996-1008.
348. Rajewsky K, Förster I, Cumano A. Evolutionary and somatic selection of the antibody repertoire in the

mouse. Science 1987;238:1088-94.
349. Griffiths GM, Berek C, Kaartinen M, Milstein C. Somatic mutation and the maturation of immune response to 2-phenyl oxazolone. Nature 1984;312:271-5.
350. Berek C, Griffiths GM, Milstein C. Molecular events during maturation of the immune response to oxazolone. Nature 1985;316:412-8.
351. Wysocki L, Manser T, Gefter ML. Somatic evolution of variable region structures during an immune response. Proc Natl Acad Sci USA 1986;83:1847-51.
352. Cumano A, Rajewsky K. Clonal recruitment and somatic mutation in the generation of immunological memory to the hapten NP. EMBO J 1986;5:2459-68.
353. Teillaud J-L, Desaymard C, Giusti AM, Haseltine B, Pollock RR, Yelton DE, Zack DJ, Scharff MD. Monoclonal antibodies reveal the structural basis of antibody diversity. Science 1983;222:721-6.
354. McKean D, Huppi K, Bell M, Staudt L, Gerhard W, Weigert M. Generation of antibody diversity in the immune response of BALB/c mice to influenza virus hemagglutinin. Proc Natl Acad Sci USA 1984;81:3180-4.
355. Clarke SH, Huppi K, Ruezinsky D, Staudt L, Gerhard W, Weigert M. Inter- and intraclonal diversity in the antibody response to influenza hemagglutinin. J Exp Med 1985;161:687-704.
356. Claflin JL, Berry J, Flaherty D, Dunnick W. Somatic evolution of diversity among anti-phosphocholine antibodies induced with *Proteus morganii*. J Immunol 1987;138:3060-8.
357. Manser T, Huang S-Y, Gefter ML. Influence of clonal selection on the expression of immunoglobulin variable region genes. Science 1984;226:1283-8.
358. Manser T, Gefter ML. The molecular evolution of the immune response: idiotope-specific suppression indicates that B cells express germ-line-encoded V genes prior to antigenic stimulation. Eur J Immunol 1986;16:1439-44.
359. Siekevitz M, Kocks C, Rajewsky K, Dildrop R. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 1987;48:757-70.
360. Kocks C, Rajewsky K. Stepwise intraclonal maturation of antibody affinity through somatic hypermutation. Proc Natl Acad Sci USA 1988;85:8206-10.
361. Sharon J, Gefter ML, Wysocki LJ, Margolies MN. Recurrent somatic mutations in mouse antibodies to *p*-azophenylarsonate increase affinity for hapten. J Immunol 1989;142:596-601.
362. Manser T. Mitogen-driven B cell proliferation and differentiation are not accompanied by hypermutation of immunoglobulin variable region genes. J Immunol 1987;139:234-8.
363. Raffeld M, Neckers L, Longo DL, Cossman J. Spontaneous alteration of idiotype in a monoclonal B-cell lymphoma: escape from detection by anti-idiotype. N Engl J Med 1985;312:1653-8.
364. Meeker T, Lowder J, Cleary ML, Stewart S, Warnke R, Sklar J, Levy R. Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotype antibodies. N Engl J Med 1985;312:1658-65.
365. Carroll WL, Lowder JN, Streifer R, Warnke R, Levy S, Levy R. Idiotype variant cell populations in patients with B cell lymphoma. J Exp Med 1986;164:1566-80.
366. Cleary ML, Meeker TC, Levy S, Lee E, Trela M, Sklar J, Levy R. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. Cell 1986;44:97-106.
367. Chien Y, Gascoigne NRJ, Kavaler J, Lee NE, Davis MM. Somatic recombination in a murine T-cell receptor gene. Nature 1984;309:322-6.
368. Goverman J, Minard K, Shastri N, Hunkapiller T, Hansburg D, Sercarz E, Hood L. Rearranged β T cell receptor genes in a helper T cell clone specific for lysozyme: no correlation between Vβ and MHC restriction. Cell 1985;40:859-67.
369. Barth RK, Kim BS, Lan NC, Hunkapiller T, Sobieck N, Winoto A, Gershenfeld H, Okada C, Hansburg D, Weissman IL, Hood L. The murine T-cell receptor uses a limited repertoire of expressed Vβ gene segments. Nature 1985;316:517-23.
370. Ikuta K, Ogura T, Shimizu A, Honjo T. Low frequency of somatic mutation in β-chain variable region genes of human T-cell receptors. Proc Natl Acad Sci USA 1985;82:7701-5.
371. Early P, Rogers J, Davis M, Calame K, Bond M, Wall R, Hood L. Two mRNAs can be produced from a single immunoglobulin μ gene by alternative RNA processing pathways. Cell 1980;20:313-9.
372. Word CJ, Mushinski JF, Tucker PW. The murine immunoglobulin α gene expresses multiple transcripts from

a unique membrane exon. EMBO J 1983;2:887-98.
373. Alt FW, Bothwell ALM, Knapp M, Siden E, Mather E, Koshland M, Baltimore D. Synthesis of secreted and membrane-bound immunoglobulin mu heavy chains is directed by mRNAs that differ at their 3' ends. Cell 1980;20:293-301.
374. Rogers J, Early P, Carter C, Calame K, Bond M, Hood L, Wall R. Two mRNAs with different 3' ends encode membrane-bound and secreted forms of immunoglobulin μ chain. Cell 1980;20:303-12.
375. Vessière-Louveaux FMYR, Hijmans W, Schuit HRE. Multiple heavy chain isotypes on the membrane of the small B lymphocytes in human blood. Clin Exp Immunol 1981;43:149-56.
376. Hijmans W, Schuit HRE, Klein F. An immunofluorescence procedure for the detection of intracellular immunoglobulins. Clin Exp Immunol 1969;4:457-72.
377. Maki R, Roeder W, Traunecker A, Sidman C, Wabl M, Raschke W, Tonegawa S. The role of DNA rearrangement and alternative RNA processing in the expression of immunoglobulin delta genes. Cell 1981;24:353-65.
378. Knapp MR, Liu C-P, Newell N, Ward RB, Tucker PW, Strober S, Blattner F. Simmultaneous expression of immunoglobulin μ and δ heavy chains by a cloned B-cell lymphoma: a single copy of the VH gene is shared by two adjacent CH genes. Proc Natl Acad Sci USA 1982;79:2996-3000.
379. Davis MM, Calame K, Early PW, Livant DL, Joho R, Weissman IL, Hood L. An immunoglobulin heavy-chain gene is formed by at least two recombinational events. Nature 1980;283:733-9.
380. Yaoita Y, Honjo T. Deletion of immunoglobulin heavy chain genes from expressed allelic chromosome. Nature 1980;286:850-3.
381. Davis MM, Kim SK, Hood LE. DNA sequences mediating class switching in α-immunoglobulins. Science 1980;209:1360-9.
382. Ravetch JV, Kirsch IR, Leder P. Evolutionary approach to the question of immunoglobulin heavy chain switching: evidence from cloned human and mouse genes. Proc Natl Acad Sci USA 1980;77:6734-8.
383. Jäck H-M, McDowell M, Steinberg CM, Wabl M. Looping out and deletion mechanism for the immunoglobulin heavy-chain class switch. Proc Natl Acad Sci USA 1988;85:1581-5.
384. Chen Y-W, Word C, Dev V, Uhr JW, Vitetta ES, Tucker PW. Double isotype production by a neoplastic B cell line: II. allelically excluded production of μ and γ1 heavy chains without CH gene rearrangement. J Exp Med 1986;164:562-79.
385. Perlmutter AP, Gilbert W. Antibodies of the secondary response can be expressed without switch recombination in normal mouse B cells. Proc Natl Acad Sci USA 1984;81:7189-93.
386. Kinashi T, Godal T, Noma Y, Ling NR, Yaoita Y, Honjo T. Human neoplastic B cells express more than two isotypes of immunoglobulins without deletion of heavy-chain constant-region genes. Genes Dev 1987;1:465-70.
387. Shimizu A, Honjo T. Immunoglobulin class switching. Cell 1984;36:801-3.
388. Radbruch A, Burger C, Klein S, Müller W. Control of immunoglobulin class switch recombination. Immunol Rev 1986;89:69-83.
389. Snapper CM, Paul WE. Interferon-γ and B cell stimulatory factor-1 reciprocally regulate Ig isotype production. Science 1987;236:944-7.
390. Coffman RL, Shrader B, Carty J, Mosmann TR, Bond MW. A mouse T cell product that preferentially enhances IgA production: I. biologic characterization. J Immunol 1987;139:3685-90.
391. Lutzker S, Rothman P, Pollock R, Coffman R, Alt FW. Mitogen- and IL-4-regulated expression of germ-line Ig γ2b transcripts: evidence for directed heavy chain class switching. Cell 1988;53:177-84.
392. Brown LA, Liu C, Berenson JR, Garcia CR, Wang R, Calame KL. Immunoglobulin JH, Cμ, and Cγ gene rearrangements in human B lymphocytes clonally transformed by Epstein-Barr virus. Proc Natl Acad Sci USA 1985;82:556-60.
393. Webb CF, Cooper MD, Burrows PD, Griffin JA. Immunoglobulin gene rearrangements and deletions in human Epstein-Barr virus-transformed cell lines producing different IgG and IgA subclasses. Proc Natl Acad Sci USA 1985;82:5495-9.
394. Borzillo GV, Cooper MD, Kubagawa H, Landay A, Burrows PD. Isotype switching in human B lymphocyte malignancies occurs by DNA deletion: evidence for nonspecific switch recombination. J Immunol 1987;139:1326-35.

CHAPTER 3.3

SOUTHERN BLOT ANALYSIS OF IMMUNOGLOBULIN GENES AND T CELL RECEPTOR GENES*

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

During rearrangements of Ig and TcR genes certain DNA segments are deleted, while other segments are relocated. These processes also change the distances between the specific cut-sites of restriction endonucleases. These are enzymes which reproducibly cut DNA only at sites where they recognize a specific sequence of base pairs; e.g. the restriction endonuclease *Eco*RI recognizes the sequence GAATTC. In the Southern blot analysis the DNA fragments (restriction fragments) produced are size-separated by agarose gel electrophoresis and subsequently transferred (blotted) onto a nitrocellulose or nylon membrane. The membrane is then incubated with a radiolabeled DNA probe, which hybridizes to complementary sequences of Ig or TcR genes. Unbound probe is washed away and the location of the probe and thereby the recognized restriction fragment is visualized by autoradiography. If the appropriate restriction enzymes and DNA probes are used, the detected restriction fragments of rearranged Ig or TcR genes will differ from those of germline genes.

The cells of most malignancies have a common clonal origin. Therefore, the cells of a lymphoid malignancy will have rearranged their Ig or TcR genes identically, while in reactive polyclonal lymphoid cell proliferations many different rearrangements of Ig and TcR genes are present. The latter rearrangements will not be detectable by Southern blot analysis, because the autoradiographic signal of each individual restriction fragment is too weak to be clearly visible within the smear of the other restriction fragments. In contrast, when a clonal cell population is studied, many identical restriction fragments will co-migrate in the agarose gel rendering their signals visible as one or more rearranged bands. Therefore Southern blot analysis of Ig and TcR genes allows discrimination between clonal rearrangements and polyclonal rearrangements (Figure 1 and Table 1).

In this chapter we shall describe the various aspects of Southern blot analysis: DNA sampling, Southern blotting itself and the various Ig and TcR gene probes. Subsequently we shall discuss several limitations of Southern blot analysis of Ig and TcR genes, such as some

---

This manuscript will be published as part of a review in Clin Chim Acta.

complicating pitfalls and the detection limit of the technique. Finally, we shall briefly indicate several diagnostic applications of Southern blot analysis of Ig and TcR genes.

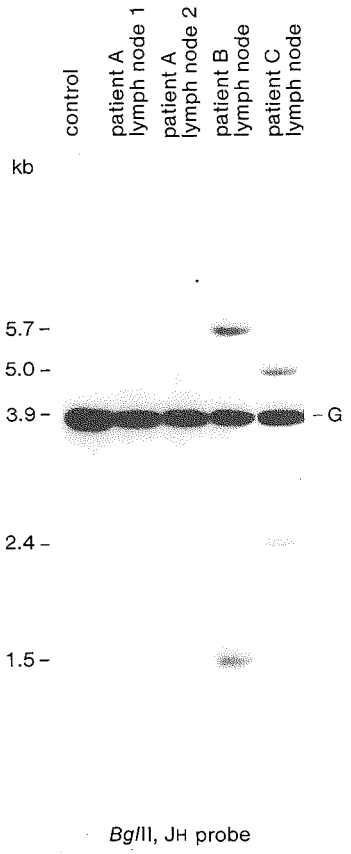


TABLE 1. Cell populations in the lymph nodes from patients A, B and C (in Figure 1) as estimated by immunologic marker analysis.

	Patient A lymph node 1	Patient A lymph node 2	Patient B lymph node	Patient C lymph node
Monoclonal B lymphocytes	0%[a]	0%[a]	10%[a]	20%[b]
Polyclonal B lymphocytes	30%[a]	50%[a]	25%[a]	25%[b]
Reactive T lymphocytes	60%	45%	50%	45%
Monocytes/ macrophages	10%	5%	15%	10%

a. The κ/λ ratios in the lymph node cell samples from patients A and B were within the normal range. Nevertheless in case of patient B a small subpopulation of $Sm\kappa^+$ large B lymphocytes could be detected.
b. In case of patient C the κ/λ ratio was slightly elevated (4.5).

Figure 1. Discrimination between a polyclonal B cell proliferation and a monoclonal B cell population by IgH gene analysis. DNA samples from two lymph node biopsies of patient A with an unexplained lymphadenopathy as well as DNA samples from lymph node biopsies of patient B and patient C were digested with the restriction enzyme *Bgl*II. The Southern blot filter was hybridized with a radiolabeled JH probe. The approximate size of the germline band (G) and rearranged bands are indicated in kilobases (kb). In the two lymph node samples from patient A no indication for the presence of a clonal B cell population was found. The background of the multiple faint non-germline bands in lymph node 1 and lymph node 2 are probably due to the presence of the reactive (polyclonal) B cells (c.f. Table 1). In case of patient B and patient C, two rearranged bands are visible in addition to the germline band. This indicates that a clonal B cell population is present in both lymph nodes. Morphological reevaluation and careful immunophenotyping revealed that 15 to 20% of the lymph node cells represented B-non-Hodgkin lymphoma (B-NHL) cells (c.f. Table 1).

DNA SAMPLING

Southern blot analysis of Ig and TcR genes to identify clonal lymphoid populations is generally performed on DNA extracted from fresh or frozen cell samples (1). In addition, several reports have described the extraction of DNA from formaldehyde-fixed and paraffin embedded tissue (2-5). Also extraction of DNA from air dried or fixed cell smears appears to be possible

(6). DNA extraction from unfixed and fixed cell samples as well as the storage of DNA samples will be discussed.

Preparation of DNA from fresh or frozen cell samples

In many cases it is possible to prepare the DNA from freshly obtained cells or frozen cells, isolated from peripheral blood (PB) or bone marrow (BM) by ficoll density centrifugation or isolated from other body fluids, such as pleural exudate and ascites. DNA is also easily extractable from freshly obtained or snap-frozen tissue samples after homogenization with a tissue grinder (e.g. from Tamson, Zoetermeer, The Netherlands; or from Kontess, Fineland, NJ). Cell pellets or homogenized tissue samples are lysed and dissolved in TNE buffer (10 mM Tris-HCl pH 7.5; 100 mM NaCl; 10 mM EDTA) at an estimated concentration of about 5 x 10^6 cells per ml. Subsequently EDTA (10 mM final concentration), Proteinase K (50 μg/ml final concentration; Merck, Darmstadt, FRG) and SDS (1% w/v final concentration) is added. This viscous mixture is incubated for at least 2 hours (or overnight) at 37°C. After this incubation DNA is extracted with an equal volume of phenol extraction buffer (50% high quality phenol; 49% chloroform; 1% isoamylalcohol) by gently mixing until a homogeneous solution is obtained. After centrifugation (5 min at 2000 g) the water phase, containing the DNA, is removed without disturbing the interphase. If admixture with interphase material occurs the extraction procedure should be repeated once or twice. To this 0.1 volume of 2 M NaAc (pH 5.6) is added, mixed and subsequently 2 volumes of cold (-20°C) ethanol (96%) are added. This is mixed until the DNA is precipitated. The precipitated DNA is removed with a small glass rod, washed in ethanol (70%) and subsequently dissolved in TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA) at 4°C; the volume of TE buffer is about 0.5 ml/5 x 10^6 cells.

When the DNA is dissolved completely, DNase-free RNase (Boehringer Mannheim, Mannheim, FRG) is added to a final concentration of 20 μg/ml and incubated for at least 1 hour at 37°C. Subsequently, Proteinase K (final concentration of 25 μg/ml) and SDS (0.1% w/v final concentration) are added and incubated for at least 2 hours at 37°C. This is followed by phenol extraction, precipitation of the DNA in NaAc and ethanol, and washing of the precipitated DNA in 70% ethanol as before. Then the DNA is dissolved in 0.1 volume TE buffer and 0.9 volume water (0.5-5 ml, depending on the amount of extracted DNA). This dissolving of the DNA generally takes 24-48 hours. Finally, the optical density of the preparations is measured with a spectrophotometer at 260 and 280 nm in order to calculate the DNA concentration and to estimate the degree of protein contamination of the sample (1).

Extraction of DNA from formalin-fixed, paraffin-embedded tissue samples

The reliance on unfixed cell samples for the isolation of DNA for Southern blot analysis limits retrospective studies, because often only fixed tissues are stored. Therefore, several investigators have developed techniques for the extraction of high molecular weight DNA from formalin-fixed, paraffin-embedded tissues (2-5).

The quality of the formalin-fixed paraffin-embedded tissues and the location of the relevant tissue areas has to be determined by means of histological sections. The paraffin blocks should not be used if the tissue appears to be fixed inadequately or if autolysis or tissue necrosis is evident, because these are important causes of failure to recover intact high-molecular mass DNA (3-5). The selected blocks of paraffin-embedded tissue are trimmed with a scalpel blade for removal of excess of paraffin wax, stromal tissue or other unwanted material (3-5). The trimmed tissue samples are diced with a scalpel or sliced with a microtome (5-μm-thick slices). The tissue slices or dices are deparaffinized and rehydrated by resuspension in xylene and subsequent resuspension and washing in xylene (once or twice), absolute ethanol (once or twice) and water (once)(see references 3 and 5). Afterwards the tissue fragments are directly transferred to 2 - 5 ml of Proteinase K digestion solution (see above) for a prolonged digestion, lasting 2 - 4 days; fresh Proteinase K (100 μg/ml final concentration) is added at 24 hours intervals (5). During this digestion the tissue fragments form a loose gelatinous lump, which disperses into fine fragments in an increasingly viscous solution. After the Proteinase K digestion, DNA is extracted with phenol extraction buffer. The subsequent steps are identical to those described above.

The rate of DNA release during the Proteinase K digestion varies in different types of tissue and depends on the density and abundance of extracellular stroma (3). In case of highly cellular tissue, such as lymphoid tissues,

most DNA is released within 1 - 2 days (3). To remove low-molecular mass DNA unsuitable for Southern blot analysis, Dubeau and colleagues (3) used a two-step Proteinase K digestion. In this method, after the first incubation (3 hours in case of lymphoid tissues) the supernatant, presumably containing only degraded DNA, is discarded. Only the supernatant obtained after the second incubation is used for DNA extraction (3). Nevertheless in all DNA samples some degraded DNA will be present.

The quality of the extracted DNA decreases progressively when the formalin fixation has lasted longer than one day (3-5). Although it has been reported that the electrophoretic mobility of restriction-enzyme-digested DNA obtained from formalin fixed tissues is decreased when compared to DNA from fresh or frozen tissues (2-5), the Southern blotting banding pattern (e.g. position of the germline bands) appears to be the same (3). Thus it can be concluded that Southern blots of DNA obtained from optimally fixed tissues (neutral buffered formalin fixation for less than 24 hours) are comparable to blots of DNA from unfixed material (3-5).

Extraction of DNA from cells of stored bone marrow or blood smears

If no fresh or frozen blood or bone marrow samples from a leukemia patient are available, the extraction of DNA from stored slides may be attempted (6). The smears are scraped off the glass slides with a scalpel blade and the resulting powdered material is transferred into an eppendorf tube. The cell material is lysed for 10 min in 1.2 ml of distilled water and 400 μl of the detergent Nonidet P40 (0.1% solution)(6). After centrifugation the nuclear pellet is incubated with the above described Proteinase K buffer. All subsequent procedures for DNA extraction are as described above.

The yield of DNA per slide is dependent on the cellularity of the cell smear: an average quantity of 16 μg DNA (range: 1-50 μg) can be obtained per bone marrow slide (6). Neither fixation nor staining of the slides seems to influence the quality of the DNA, because DNA from unfixed, methanol-fixed, and stained slides appeared to give comparable results in Southern blot analysis (6).

Generally, the DNA extracted from slides is partially degraded, and it should be emphasized that the quality of the analysis is unlikely to be optimal (6). Especially large restriction fragments (> 10 kb) may appear to be absent.

Control DNA samples

For optimal Southern blot analysis of Ig and TcR genes control DNA samples are needed to determine the exact position of the germline bands. In principle DNA from any cell sample with germline Ig and TcR genes can be used for this purpose. Frequently used sources for such germline DNA are non-hematopoietic cells, such as placenta, fibroblasts, liver cells, carcinomas and cell lines (7-10). Also DNA from malignant T cells with germline Ig genes or DNA from malignant B cells with germline TcR genes can be used as controls for the analysis of Ig genes and TcR genes, respectively.

More or less extensive polymorphisms occur in many genes. Some of them involve the target sequences of restriction enzymes, which results in restriction fragment length polymorphisms (RFLP). A second type of DNA polymorphism, designated hypervariable (or length) polymorphism (HVP), results from insertion or deletion of DNA segments (11). Also polymorphic amplification of genes or gene segments may occur (12,13). Although only a few RFLP, HVP and polymorphic gene amplifications occurring in Ig genes (11,12,14-17) and TcR genes (13,18-24) have been reported, it may be important to obtain both the tumor DNA and the control DNA from the same patient. In retrospective studies it will often be difficult to collect control DNA from the same patient as the tumor DNA. On the other hand, in prospective studies one should try to obtain granulocytes or fibroblasts from each patient.

Granulocytes represent an easily-accessible source of control DNA. The nuclei of purified granulocytes can be obtained after ficoll density centrifugation of peripheral blood by lysis of the erythrocyte/granulocyte pellet with a detergent such as Zaponin (Coulter Electronics, Luton, UK) and subsequent washing with phosphate buffered saline. Cultured fibroblasts from a skin biopsy may also serve as a source of control DNA, but usually several weeks of culture are required to obtain sufficient numbers of cells.

Storage of DNA samples

DNA samples are often stored at a concentration of 100-400 μg/ml in an eppendorf tube or in a larger well-sealed tube at 4°C. This is suitable for short-term storage, but long-term storage (> 1 year) at 4°C results in a progressive degradation of the DNA and, sometimes, infection may develop. Therefore in our laboratory long-term storage of DNA samples is performed in eppendorf tubes at -70°C. This prevents degradation of the DNA as well as growth of microorganisms. The aliquots of DNA per tube should not exceed 200-400 μg, since repeated freezing and thawing will lead to progressive shearing of the DNA.

SOUTHERN BLOT ANALYSIS

In this section we shall describe the digestion of DNA by use of restriction enzymes, the agarose gel electrophoresis of the restriction fragments, the blotting of the fragments onto a membrane, hybridization with radiolabeled DNA probes and the autoradiography (1,25). Several aspects of these procedures are illustrated in Figure 2.

Restriction enzyme digestion

Approximately 15 μg of DNA is digested with 100 units of the appropriate enzyme (Pharmacia, Uppsala, Sweden; Boehringer Mannheim, Mannheim, FRG; New England Biolabs, Beverly, MA; Amersham, Amersham, UK; Promega, Madison, WI). The most suitable type of digestion buffer (low, medium, or high ionic strength) depends on the type of restriction enzyme used and should be prepared according to the manufacturers guidelines. The complete digestion mixture contains 15 μg of DNA, 100 units restriction enzyme, 20 μl of 10x concentrated digestion buffer, 1 μl of 20% BSA (special quality for molecular biology; Boehringer Mannheim) and 8 μl of 100 mM Spermidine (Sigma, St. Louis, MO) in case of high ionic digestion buffer; this mixture is supplemented with distilled water to a final volume of 200 μl. The digestion mixture is incubated at 37°C for 6 hours (or overnight) in an eppendorf tube while shaking slowly.

Two controls are performed per digestion in order to assess its completeness. These include 10 μl of the digestion mixture supplemented with ½ μg of bacteriophage λ DNA as first control and 10 μl digestion mixture supplemented with ½ μg of plasmid DNA containing an insert with many different restriction enzyme sites as second control. These control digests are incubated overnight at 37°C in eppendorf tubes together with the sample digests. After the incubation the control digests are evaluated for completeness of digestion as follows. One volume of TES buffer (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.1% SDS) is added to both control tubes. Subsequently two volumes of phenol extraction buffer are added. After mixing and centrifugation (3 min at 15,000 g in a high-speed microcentrifuge; Centra-M, International Equipment Company, Needham, MA) the water phases, completed with 5 μl of loading buffer with Orange G as tracking dye (20% ficoll; 10 mM Tris-HCl, pH 7.5; 0,1% Orange G), are run in a 0.7% agarose gel in TAE buffer (40 mM Tris; 10 mM EDTA; adjusted to pH 8.2 with glacial acetic acid), containing ethidium bromide (0.5 μg/ml; Serva Fein Biochimica, Heidelberg, FRG) to stain the DNA. After running the gel at 80-100 V for 1-2 hours, the resulting banding pattern is visualized with UV light (~300 nm)(Figures 2A and 2B). If the banding pattern indicates that the digestion is complete, it is assumed that also the digestion of the human DNA sample is complete. If the digestion of the λ DNA and/or plasmid DNA appears to be incomplete, additionally 100 units of restriction enzyme are added to the human DNA digestion mixture, followed by a second incubation for a few hours or overnight (depending on the degree of incompleteness of digestion). Also this second digestion is checked by parallel incubations with λ DNA and plasmid DNA.

When the digestion of the human DNA sample is assumed to be complete, a phenol extraction is performed by adding SDS (0.1% final concentration), EDTA (5 mM final concentration) and an equal volume of phenol extraction buffer to the eppendorf tube. After centrifugation (3 min at 15,000 g) the water phase is isolated and the DNA is precipitated by adding 0.1 volume of 2 M NaAc (pH 5.6) and 2 volumes of ethanol (96%)(precipitation

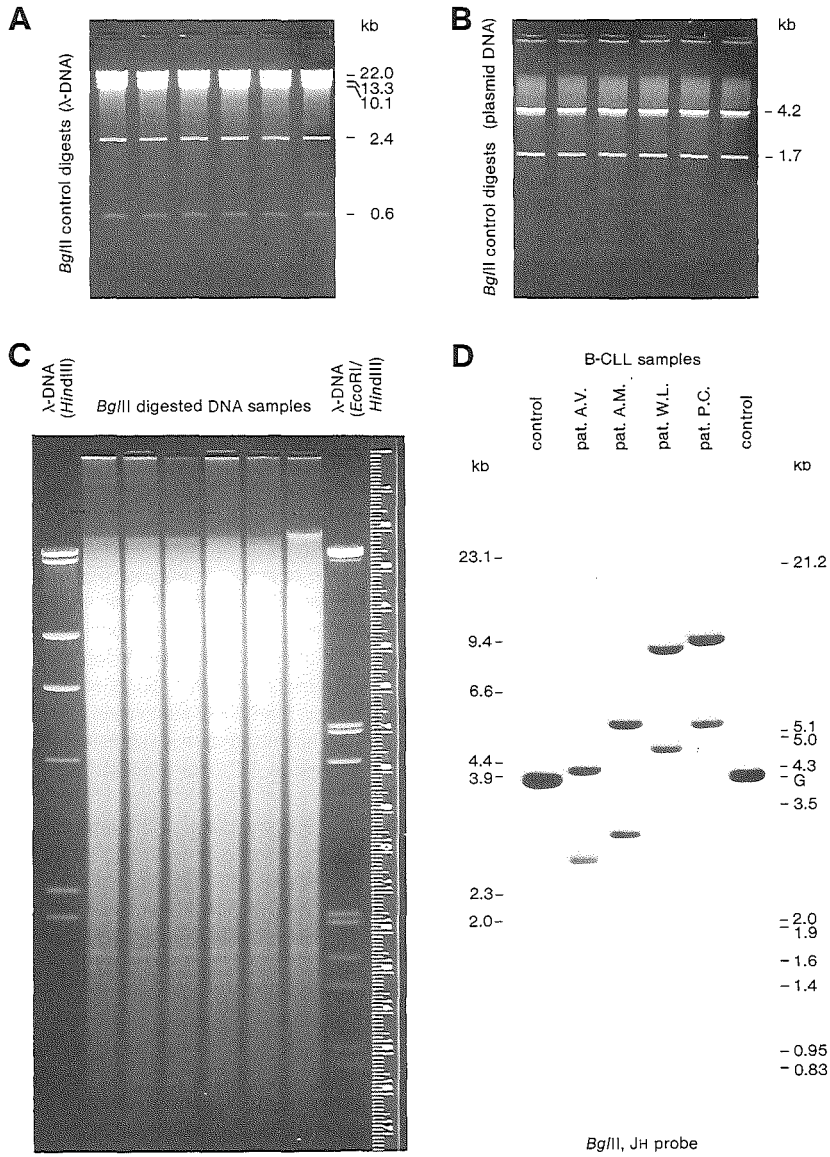


Figure 2. Southern blot analysis of the IgH genes of a control DNA sample (T cell leukemia) and four different B cell chronic lymphocytic leukemia (B-CLL) samples by use of the restriction enzyme *Bgl*II and the JH probe. **A**:*Bgl*II control digests supplemented with λ-DNA. **B**: *Bgl*II control digests supplemented with plasmid DNA. **C**: Ethidiumbromide stained agarose gel with size-separated *Bgl*II restriction fragments of the control DNA sample and the four B-CLL samples. The two outer lanes contain size markers: *Left*: *Hin*dIII digested λ-DNA; *Right*: *Eco*RI/*Hin*dIII digested λ-DNA. **D**: Photograph of the X-ray film after exposure to the *Bgl*II filter, which was hybridized with a radiolabeled JH probe. The approximate sizes of the germline band (G) and the rearranged bands are indicated in kilobases (kb).

overnight at -20°C or for 30 min at -70°C). After centrifugation (15 min at 15,000 g) the pellet is washed in ethanol (70%), centrifuged again and subsequently air-dried to evaporate all ethanol. The pellet is then dissolved in 20 µl H_2O and 5 µl loading buffer with Orange G as tracking dye.

Agarose gel electrophoresis

The agarose used for the electrophoretic separation of DNA restriction fragments should be of high quality (FMC Rockland, ME; Sigma). The concentration of the agarose depends on the expected sizes of the restriction fragments. Generally 0.7% agarose gels are used when one expects relatively small restriction fragments (1-15 kb), while 0.5-0.6% agarose gels are used when one expects larger restriction fragments (15-25 kb). If possible, the analysis of restriction fragments of > 20 kb should be avoided, because of the difficulty in separating such large fragments in conventional gel electrophoresis. For this purpose pulsed field gradient gel electrophoresis is recommended (26-29); this technique will not be discussed here.

Several types of well designed plexiglas electrophoresis equipment are commercially available (e.g. from DanKar Corporation, Reading, MA; or from Pharmacia). These usually consist of an electrophoresis tank with an accompanying gel tray and several types of slotformers. The gel tray can be used for both pouring as well as running the gel, and allows a precise and reproducible placing of the slot formers. The top and the bottom side walls of the gel tray are carefully sealed and the slot former is placed at the appropriate position.

The agar is dissolved in TAE electrophoresis buffer, containing ethidium bromide (0.5 µg/ml), by bringing it to boil in a microwave oven. The agarose is then cooled to approximately 50°C before pouring the gel in order to prevent the opening of the seams of the plexiglas gel tray. It is important that the gel tray is placed at a precisely horizontal position during pouring and congealing of the gel. After the gel is completely set (30-45 min at room temperature) the slot formers and the tray seals are carefully removed. The gel tray is submerged in the electrophoresis tank; the TEA electrophoresis buffer should be sufficient to cover the gel with a fluid layer of 3-5 mm.

The digested DNA samples can now be loaded into the slots of the gel; the two outer slots are used to load λ-DNA restriction fragments as size markers. If the expected sizes of the restriction fragments are between 1 and 15 kb, one should use *Hin*dIII-*Eco*RI digested λ-DNA and *Hin*dIII digested λ-DNA as size markers (Figures 2C and 3). If the expected restriction fragments are between 15 and 25 kb, also other λ-DNA restriction fragments should be used, e.g. *Stu*I, *Xho*I, *Xho*I/*Nae*I or *Xho*I/*Kpn*I digested λ-DNA (Figure 3).

Electrophoresis is performed at 30-40 V overnight (about 15 hours) with a precisely horizontal position of the tank. When the Orange G tracking dye has run for 18-20 cm, the electrophoresis is stopped and the gel is inspected by use of transillumination with UV light of ~300 nm (TM20 UV-transilluminator; UVP, San Gabriel, CA). Photographs of the gel can be made with a polaroid camera (Polaroid MP-4 Land-Camera; Polaroid, Cambridge, MA) or a reflex camera with macro-objective (Nikon FM2 camera with an E2 focusing screen and a 55 mm micro-Nikkor 2.8 objective; Nikon, Tokyo, Japan) using Polaroid 667 film for the polaroid camera or Ilford PAN F 50 ASA film for the reflex camera (1). During photography a UV ruler (Diversific Biotech, Newton Centre, MA) is placed along the gel for future estimation of the sizes of rearranged and germline bands (Figure 2C).

Blotting of the restriction fragments to a membrane

After size fractionation, the restriction fragments are blotted onto a nitrocellulose membrane or a nylon membrane (1,25). We prefer nylon membranes because of their high mechanical strength and chemical resistance, which permit both the use of highly stringent assay conditions and repeated hybridizations. Several types of nylon membranes are commercially available (e.g. Nytran 13N membranes from Schleicher and Schuell, Dassel, FRG; Gene Screen nylon membranes from New England Nuclear, Boston, MA; Biodyne nylon membranes from Pall Ultrafine Filtration Corporation, Glen Core, NY). The nylon membrane is wetted thoroughly in distilled water and subsequently soaked in transfer buffer until use.

In order to achieve an even transfer of small and large restriction fragments, the DNA is fragmentated before blotting by an acid and a subsequent alkaline treatment of the gel (1,25). First, the gel is immersed in 0.25 M HCl for 10 min at room temperature, while shaking gently. Second, the gel is soaked twice in the alkaline solution (1.0

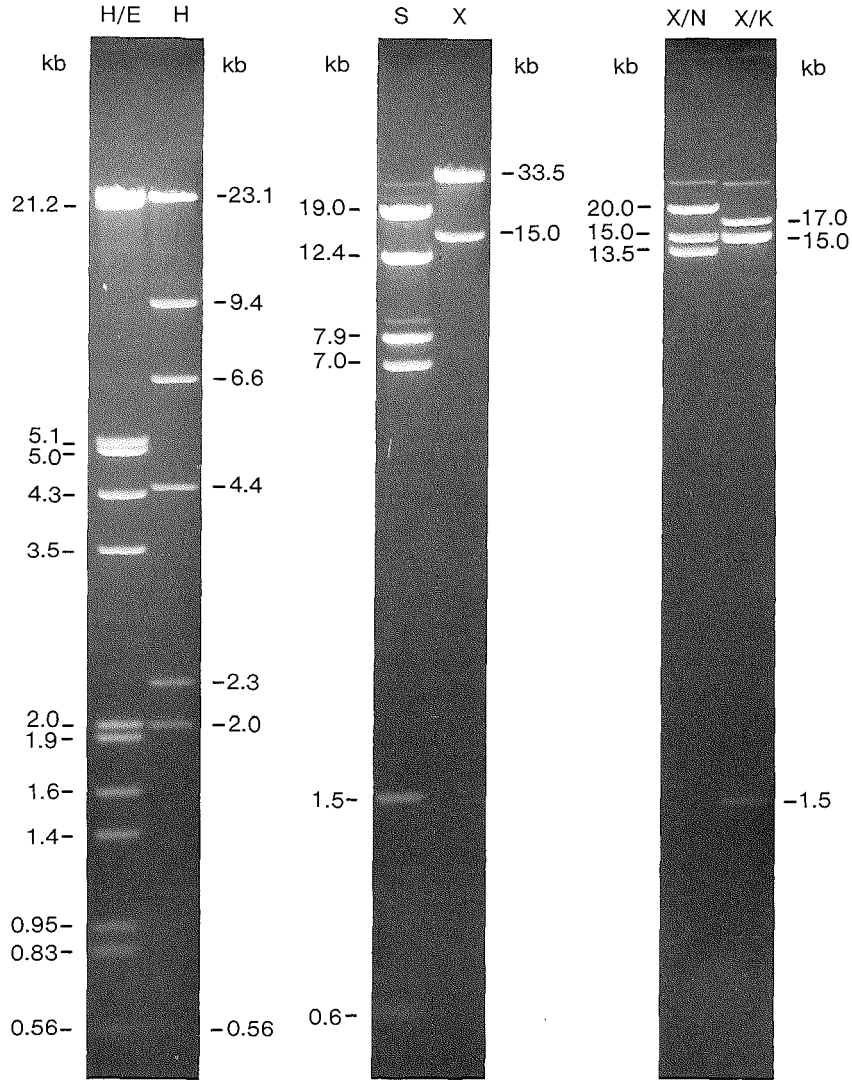


Figure 3. Size markers for agarose gel electrophoresis of restriction fragments. *Hin*dIII/*Eco*RI (H/E) digested λ-DNA and *Hin*dIII (H) digested λ-DNA can be used as size-markers for calculation of the sizes of restriction fragments between 1 and 15 kb. *Stu*I (S) digested λ-DNA, *Xho*I (X) digested λ-DNA, *Xho*I/*Nae*I (X/N) digested λ-DNA, and *Xho*I/*Kpn*I (X/K) digested λ-DNA can be used as size markers for calculation of the sizes of restriction fragments between 15 and 25 kb.

M NaCl; 0.5 M NaOH) for 15 min at room temperature while shaking gently. The alkaline solution also denatures the DNA, which is necessary for the subsequent hybridization procedure. After the alkaline treatment the gel is neutralized by soaking in neutralization buffer (0.5 M Tris, pH 7.4; 1.5 M NaCl) for 2 x 20 min while shaking gently. Finally the gel is soaked for 15 min in transfer buffer (10 x SSC: 1.5 M NaCl; 150 mM $C_6H_5Na_3O_7$, pH 7.0).

The fragmentated DNA is transferred to the nylon membrane by the capillary blotting method via the mass flow of transfer buffer through the agarose gel to the absorbent layer of paper on the top of the gel (1,25). For this purpose a piece of GB004 blot paper (Schleicher and Schuell) is placed on a glass plate, the sides of the blot paper being draped into the transfer buffer reservoir. The gel is placed on the blot paper, followed by the prepared nylon membrane and eight layers of 3 mm Chr Whatmann paper (Whatmann, Maidstone, UK), which are cut to fit the gel and soaked in the transfer buffer. A 5-10 cm stack of paper towels is placed on top, followed by a glass plate and a weight of 1/2 - 1 kg to secure the blot. During the set-up of the blot all air-bubbles should be carefully removed from each layer by rolling a glass rod over the surface.

The blotting requires 2-4 hours at room temperature, but is generally carried out overnight. After the blotting the nylon membrane is washed in 10 x SSC, air dried for 2 hours and baked in an oven at 80°C for 2 hours.

Hybridization with ^{32}P-labeled DNA probes

Hybridization of the membrane-bound DNA involves three essential steps: A, prehybridization to block non-specific hybridization of the probe to the membrane; B, hybridization with the ^{32}P-labeled probe; C, stringent washes to eliminate non-specific binding of the probe (1,30).

The nylon membrane is first thoroughly wetted in 0.5 M $NaHPO_4$. Prehybridization with 50 ml hybridization mixture is carried out in a plastic box with a lid at 65°C for 1/2 to 1 hour. The hybridization mixture consists of: 0.5 M $NaHPO_4$ pH 7.2, 1% BSA Fraction V (Boehringer Mannheim), 1 mM EDTA, 3% SDS and 200 µg/ml sheared salmon sperm DNA.

Subsequently, the ^{32}P-labeled genomic Ig or TcR probe (see next section) is added to the mixture. The probe has to be denatured before use by boiling in TES buffer for 3 min and immediate cooling down on ice. The hybridization is performed overnight at 65°C, while shaking gently. During the prehybridization and the hybridization, the membrane(s) should be submerged completely and no air-bubbles should be present under or between the membrane(s).

After hybridization the membrane is washed once in wash buffer 1 (40 mM $NaHPO_4$, pH 7.2; 2% SDS; 1 mM EDTA; 0.5% BSA) for 5 min at 60-65°C, followed by eight washes in wash buffer 2 (40 mM $NaHPO_4$, pH 7.2; 1% SDS; 1 mM EDTA) for 3-5 min at 60-65°C. Subsequently the membrane is rinsed with 100 mM $NaHPO_4$ (pH 7.2) and the excess of buffer is removed by briefly placing the membrane between two paper layers. Finally the membrane is sealed in a plastic bag, ready for X-ray film exposure.

Film exposure

The sealed membrane is placed in a cassette with an X-ray film and an intensifying screen. For this purpose we generally use Fuji NIF-RX films (Fuji Photo Film Co., Japan) or the more sensitive Kodak X-Omat AR films (Eastman Kodak Company, Rochester, NY). The exposure is performed at -70°C for 1 - 4 days. The optimal exposure time depends on the type of DNA probe and the quality of the ^{32}P-labeling, and should be determined empirically.

Probe removal for rehybridization

Hybridized DNA probes can be removed easily from nylon membranes without loss of the transferred DNA fragments. This enables repeated hybridizations of a single membrane with different DNA probes (30). This may be important when only a restricted quantity of DNA is available.

The membrane is washed in 50% formamide and 6 x SSC for 30 min at 65°C, rinsed in 3 x SSC, soaked in 0.1 M $NaHPO_4$, dried and sealed in a plastic bag for storage or directly used for rehybridization. In the latter case an X-ray exposure may be performed in order to exclude the presence of residual ^{32}P-labeling on the membrane.

ISOLATION AND RADIOLABELING OF PROBES

The DNA probes used for Southern blot analysis of the Ig and TcR genes are genomic sequences, complementary to segments directly involved in the rearrangements of these genes. The majority of the probes are available as inserts in plasmids.

Plasmids are extrachromosomal, circular, double-stranded DNA molecules, which can be artificially transferred into bacteria. This process is called transformation. The various types of laboratory plasmids contain genes coding for selection markers, such as resistance to antibiotics (e.g. ampicillin and tetracycline) as well as a DNA segment with several closely spaced restriction sites (the so-called polylinker) into which foreign DNA (e.g. Ig or TcR probes) can be inserted (1).

Plasmid-transformed bacteria allow a large scale growth of the plasmids, which can subsequently be isolated. The inserts are cut out, purified, radiolabeled and used as probe for Southern blot analysis. Several aspects of the isolation of probes will be described.

Transformation of bacteria with plasmids

Plasmid transformation of Escherichia coli (E. coli) can be performed in the presence of divalent cations (e.g. $CaCl_2$) at low temperatures (31). A variety of factors may improve the transformation frequency obtained with divalent cations alone, e.g. brief exposure of the bacteria-plasmid mixture at 42°C (32).

Freshly isolated *E. coli* colonies grown with $MgCl_2$ are dissolved in 150 µl of 100 mM $CaCl_2$ at 4°C for 2 hours in an eppendorf tube. Subsequently 50 µl of 100 mM $CaCl_2$ with 20-50 ng of plasmid (containing the appropriate insert) are added to the bacteria. This mixture is first incubated for 30 min at 4°C, then for 1/2 to 1 min at 42°C and finally for 2 min at 4°C. Next, 800 µl of SOC-medium are added, followed by an incubation for 45 min at 37°C. SOC-medium consists of 2% (w/v) Bacto-tryptone (Difco, Detroit, MI), 0.5% (w/v) Bacto-yeast extract (Difco), 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$ and 20 mM glucose. The mixture is then centrifuged (30 seconds at 15,000 g), the supernatant is decanted and the bacteria pellet is resuspended in the remaining medium. The bacteria are plated and cultured overnight on L-Broth agar, supplemented with antibiotics (e.g. ampicillin or tetracycline, depending on the resistance of the plasmid). L-Broth agar consists of 1% Bacto-tryptone, 0.5% Bacto-yeast extract, 0.5% NaCl and 1.5% Bacto-agar (Difco), adjusted to pH 7.2 with NaOH. If the transformation is successful, colonies will be visible after an overnight culture at 37°C.

Evaluation of plasmid transformation

To evaluate whether the growing colonies have been transformed correctly, six colonies are picked and grown in 5 ml L-Broth medium (same composition as L-Broth agar, but without agarose) at 37°C overnight. A part of each culture (450 µl) is supplemented with 50 µl of 10 x Hogness (36 mM K_2HPO_4; 13 mM KH_2PO4; 20 mM $C_6H_5Na_3O_7$; 10 mM $MgSO_4$; 44% glycerol) and stored at -70°C.

The remaining of each culture is tested for the presence of the plasmid with insert as follows. After centrifugation (5 min, 2000 g) bacteria are lysed in 200 µl Maxiprep (50 mM glucose; 25 mM Tris-HCl, pH 7.5; 10 mM EDTA), completed with 400 µl of 0.2 M NaOH and 1% SDS. Subsequently, 200 µl of 3 M NaAc (pH 4.8) is added in order to precipitate the bacterial DNA. After centrifugation (3 min, 15,000 g) the supernatants with the plasmid DNA are collected and 0.5 ml of isopropanol is added to precipitate the plasmid DNA. The tubes are centrifuged again (3 min, 15,000 g), the supernatants are discarded and the pellets are washed in ethanol (70%), air-dried and dissolved in 200 µl distilled water. Subsequently RNase is added to a final concentration of 200 µg/ml, followed by an incubation at 37°C for 30 min. Then EDTA (5 mM final concentration) and SDS (0.1% final concentration) are added to each tube and a phenol extraction is performed (see above). The water phases are

isolated and a NaAc/ethanol precipitation is performed by adding 0.1 volume of NaAc (2 M, pH 5.6) and 2 volumes of cold (–20 °C) ethanol (96%). After centrifugation the pellets are washed in ethanol (70%), air-dried and dissolved in 100 µl distilled water.

About 20 µl of each isolate is mixed with 0.1 volume of 10 x digestion buffer (the type of digestion buffer depends on the type of restriction enzyme; see above) and 10 units of the appropriate restriction enzyme is added to cut out the inserts from the plasmids. After an incubation of 2-4 hours at 37 °C, EDTA (5 mM final concentration) and SDS (0.1% final concentration) are added, followed by a phenol extraction. The water phases of the six tubes are mixed with 5 µl loading buffer with Orange G as tracking dye and run on a 0.7% agarose gel, containing 0.5 µg/ml ethidium bromide. *Hin*dIII-*Eco*RI digested λ-DNA is used as size marker. After running the gel at 80 - 100 V for 1-2 hours the gel is inspected by use of transillumination with UV light (300 nm). If the transformation is successful and if the plasmid isolation as well as the restriction enzyme digestion have been performed properly, in most cases two bands will be visible: a plasmid band and an insert band.

Large scale growth of plasmids

A culture of the plasmid-transformed bacteria colony is started in 5 ml of L-Broth medium, supplemented with antibiotics (selected as the basis of the resistance of the plasmid) and incubated at 37 °C for 3-4 hours while shaking. This culture is transferred to one liter of L-Broth medium with antibiotics and incubated overnight at 37 °C while shaking (1).

Nine ml of the large scale culture are mixed with 1 ml of 10 x Hogness and stored at -70 °C in 1 ml vials for future cultures. The remaining of the culture is centrifuged (10 min, 5000 g). The bacteria-pellet is lysed in a mixture of 40 ml Maxiprep (50 mM glucose; 25 mM Tris-HCl, pH 7.5; 10 mM EDTA) and 80 ml of 0.2 M NaOH and 1% SDS. After mixing gently, the bacterial DNA is precipitated by adding 40 ml of 3 M NaAc (pH 4.8). After centrifugation (10 min at 5000 g) the supernatant is mixed with 0.6 volume of isopropanol to precipitate the plasmid DNA.

The precipitated plasmid DNA is centrifuged (10 min, 5000 g) in a Sorvall centrifuge (Sorvall RC5C with GSA rotor; Du Pont Co., Wilmington, DE). The pellet is air-dried and dissolved in 9 ml of 10 x TE buffer (100 mM Tris-HCl, pH 7.5; 10 mM EDTA). Subsequently 10.2 g of cesium chloride and 1 ml of ethidium bromide (5 mg/ml) is added. This mixture is transferred to a special ultracentrifuge tube (e.g. 11.5 ml Ultracrimp sealable tube; Du Pont) for overnight centrifugation in a Sorvall OTD75B Ultracentrifuge with a T1270 rotor (Du Pont) at 20 °C and a rotor speed of 50,000 rpm. After centrifugation the top of the tube is performed to admit the entrance of air. Subsequently, the lower band, containing the circular plasmid DNA, is collected by puncturing the side of the tube just below the plasmid band by use of a 21-gauge needle; contamination of the plasmid DNA with material from other bands should be avoided. One volume of distilled water and two volumes of ethanol (96%) are added to precipitate the plasmid DNA (during 15 - 30 min). After centrifugation (10 min; 2000 g), the pellet is washed in ethanol (70%), air-dried and dissolved in 500 µl distilled water containing EDTA (5 mM final concentration) and SDS (0.1% final concentration), followed by a phenol extraction. The water phase is transferred to an eppendorf tube and a NaAc-ethanol precipitation is performed by adding 0.1 volume 2 M NaAc (pH 5.6) and 2 volumes of cold (-20 °C) ethanol (96%). After centrifugation (5 min at 15,000 g) the pellet is washed in ethanol (70%), air-dried and dissolved in 500 µl distilled water. Then RNase is added to a final concentration of 200 µg/ml, followed by an incubation of 30 min at 37 °C. Afterwards EDTA (5 mM final concentration) and SDS (0.1% final concentration) are added, followed by a phenol extraction. The plasmid DNA in the water phase is precipitated by use of 0.1 volume of 2 M NaAc (pH 5.6) and 2 volumes of cold (-20 °C) ethanol (96%). After centrifugation the pellet is washed in ethanol (70%), air-dried and dissolved in 100-400 µl TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA).

Isolation of inserts from plasmids

Approximately 50 µg of plasmid DNA (with the appropriate Ig or TcR probe as insert) is mixed with 0.1 volume of 10 x digestion buffer (selected according to the type of enzyme) and 50 to 100 units of the appropriate restriction enzyme to cut out the insert from the plasmid. The final volume is usually ~100 µl. This mixture is incubated at 37 °C for 3-4 hours or overnight.

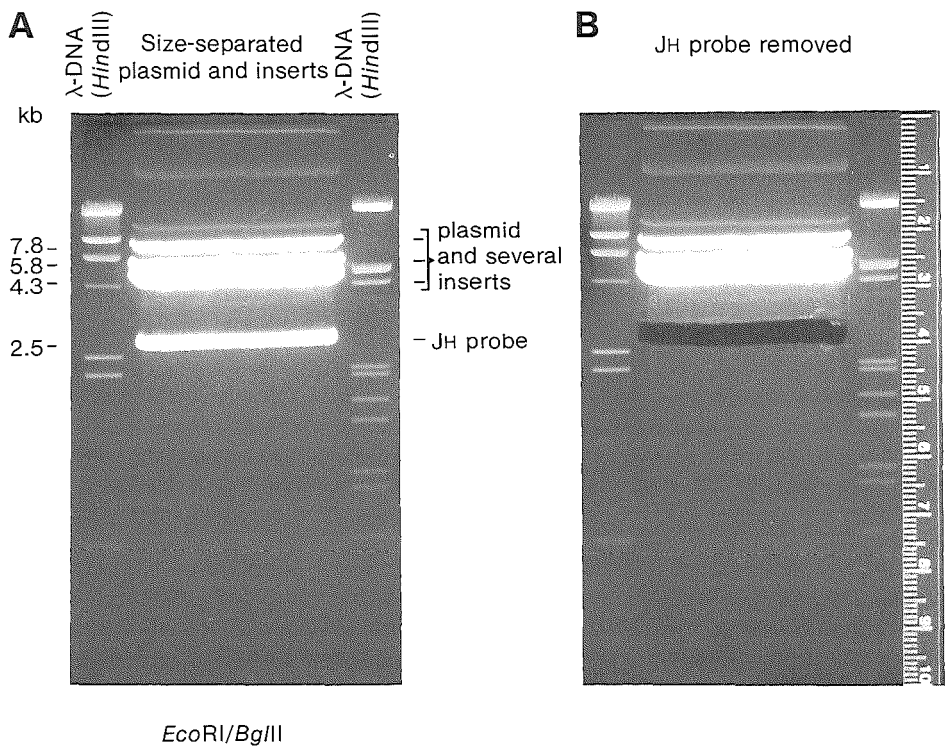


Figure 4. Isolation of the human JH probe. After digestion with the restriction enzymes *Eco*RI and *Bgl*II the plasmid (pBR328) and insert fragments (including the JH probe) are separated in an 0.7% agarose gel. **A**: Ethidium bromide stained agarose gel of size-separated plasmid and insert fragments. **B**: The band containing the JH probe (2.5 kb) is cut out of the gel.

In order to evaluate the degree of restriction enzyme digestion, 2 μg of plasmid is taken from the incubation mixture and added to 16 μl of TES buffer (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.1% SDS) and 20 μl of phenol extraction buffer. After the phenol extraction, the water phase is mixed with 5 μl loading buffer (containing Orange G) and loaded in a 0.7% agarose gel (containing 0.5 μg/ml ethidium bromide to stain the DNA). After running the gel for 1-2 hours at 80 - 100 V the banding pattern is evaluated by use of UV light. If the digestion appears to be incomplete, additional restriction enzyme is added to the incubation mixture and the incubation is prolonged for several hours (depending on the degree of incompleteness of digestion) as already discussed.

If the digestion is complete, EDTA (5 mM final concentration) and SDS (0.1% final concentration) are added to the digestion mixture and a phenol extraction is performed. The water phase (~100 μl) is mixed with 25 μl loading buffer (containing Orange G) and loaded into a broad slot (about 3-5 cm) of a 0.7% agarose gel. Both the gel and the electrophoresis buffer contain 0.5 μg/ml ethidium bromide. Electrophoresis is performed at 80 - 100 V until the plasmid band and the insert band are sufficiently separated to cut out the latter band with a sterile scalpel blade (Figure 4). The probe is electrophoretically recovered from the agarose gel fragment by using a separation chamber specially designed for this purpose (Biotrap; Schleicher and Schuell) at 100 V overnight. The concentration of the probe is estimated with ethidium bromide staining by comparing it to standard DNA concentrations using UV light.

Radiolabeling of DNA probes

DNA probes are usually radiolabeled by either nick-translation (33,34) or the Klenow-oligonucleotide method (35).

In the *nick-translation method* E. coli DNA polymerase I is used to replace preexisting nucleotides with radioactive nucleotides (33,34). E. coli DNA polymerase I, by virtue of its 5´ to 3´ polymerase activity, adds nucleotides to the 3´ hydroxyl terminus that is created when one strand of a double-stranded DNA molecule is nicked (e.g. with DNase I). E. coli DNA polymerase I can also remove nucleotides from the 5´ side of the nick, due to its 5´ to 3´ exonucleolytic activity. The elimination of nucleotides from the 5´ side and the sequential addition of nucleotides to the 3´ side results in the movement of the nick along the DNA (nick translation). It is therefore possible to prepare radiolabeled probes by replacing the preexisting nucleotides with ^{32}P-labeled nucleotides.

The *Klenow-oligonucleotide method* is based on the fact that oligonucleotides can serve as primers for copying single stranded templates in the presence of DNA polymerase (35). In the large fragment of E. coli DNA polymerase I (the Klenow fragment) the 5´ to 3´ polymerase activity is preserved, while the 5´ to 3´ exonucleolytic activity is lost, preventing the degradation of oligonucleotides. Therefore, in the presence of oligonucleotides (e.g. hexadeoxyribonucleotides) Klenow fragments allow the copying of DNA probes, after they have been denatured (e.g. by boiling). If ^{32}P-labeled nucleotides are added during the process, all copies of the probe will be radiolabeled.

In our laboratory, we prefer to use the Klenow-oligonucleotide method for ^{32}P-labeling of DNA probes, because even one or two ^{32}P-labeled deoxynucleotidetriphosphates (dNTP) are sufficient to obtain highly labeled DNA probes. In contrast all four types of dNTP have to be ^{32}P-labeled when using the nick-translation method. An additional advantage of the Klenow-oligonucleotide method is that over 70% of the ^{32}P-dNTP will be routinely incorporated into the probe (35).

The *Klenow-oligonucleotide method* is performed as follows. Eleven µl of distilled water containing about 50-100 ng of DNA probe are boiled for 3 min to denature the probe and cooled down on ice. Subsequently 4 µl of oligo-labeling-buffer (OLB), which contains unlabeled dGTP and dTTP as well as oligonucleotides (see reference 35 for a detailed description of the composition of OLB), is added together with 0.8 µl of 20% BSA (Boehringer Mannheim), 2 µl of (α^{32}P)-dATP, 2 µl of (α^{32}P)-dCTP (Amersham) and 2-4 units of Klenow (Promega). This mixture is incubated for 3-5 hours at room temperature. The reaction is stopped by adding 80 µl of TES buffer (10 mM Tris-HCl, pH 7.5; 5 mM EDTA; 0.1% SDS).

The radiolabeled probe is separated from the unincorporated dNTP on a small Sephadex G-50 column (1). For this purpose the bottom of a 1 ml disposable syringe is plugged with a small amount of sterile glass wool and subsequently 0.9 ml of Sephadex G-50 (Pharmacia) equilibrated in water is added. The needle of the syringe is pricked through the cap of an eppendorf tube (the cap should have additional perforations to let out air during centrifugation). The total column is placed in a glass tube (Corex tube, Du Pont) and centrifuged for 1 min at 500 g. This removes the excess of water and packs the Sephadex. Extra Sephadex is added, followed by centrifugation, until the packed column reaches the 0.9 ml mark. After replacing the eppendorf tube the reaction mixture is loaded onto the column and centrifuged (1 min at 600 g). Additional 100 µl of TES buffer might be loaded and centrifuged for further probe recovery.

The ^{32}P-labeled DNA probe in the eppendorf tube is boiled for 3 minutes and subsequently cooled down on ice. Now the probe is ready for hybridization.

IMMUNOGLOBULIN PROBES AND T CELL RECEPTOR PROBES

Since J gene segments are usually involved in the rearrangements of Ig and TcR genes, these can be detected by using probes complementary to the 3′ side of the J regions. The use of C region probes can also be informative, when the recognized sequences reside on the same restriction fragment as the J gene segments (see below). C region probes may also give valuable information about changes in C regions, such as IgH class switch rearrangements or deletions.

When Ig or TcR gene analysis is performed for diagnostic purposes, the investigation of IgH and/or TcR-β genes is usually sufficient. In more complicated cases the analysis of the IgL genes and/or TcR-γ and TcR-δ genes may be necessary. The analysis of the TcR-α gene complex is difficult, because more than ten Jα probes may be needed to analyse the complete Jα region (36,37), which consists of a long stretch (> 80 kb) of about 55 Jα gene segments (38-40). The availability of detailed information about the germline configuration of the genes and the position of the restriction sites is a prerequisite for an accurate study. In addition the appropriate probes must be available. We shall summarize this information for the IgH, Igκ and Igλ genes (Figures 5-11 and Tables 2 and 3) as well as for the TcR-β, TcR-γ and TcR-δ genes (Figures 12-15 and Table 4).

IMMUNOGLOBULIN PROBES

IgH genes

Rearrangements of the IgH genes are detectable with a JH probe (Figure 5) after digestion with the restriction enzyme *Bgl*II, *Bam*HI or the combination of *Bam*HI and *Hin*dIII. The interpretation of results obtained with the enzymes *Hin*dIII or *Eco*RI might be difficult, because a hypervariable polymorphic (HVP) region is located between the JH region and the first *Hin*dIII and *Eco*RI sites at the 5′ side of the JH region (see below; 11). Data about the germline con-

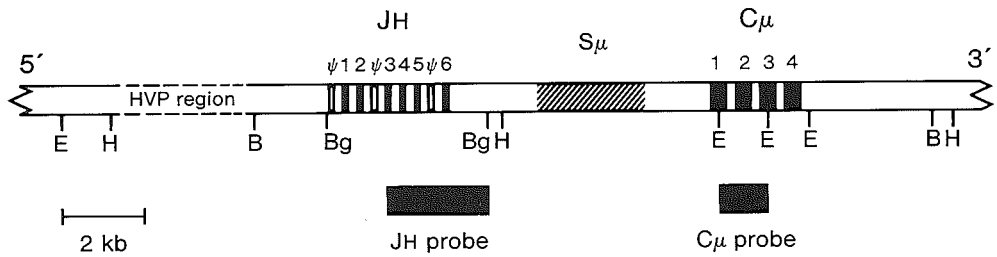


Figure 5. Organization of the J and Cμ regions of the human IgH genes (references 11,41-44). The locations of the relevant *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B) and *Bgl*II (Bg) restriction sites are indicated. Also the location of the hypervariable polymorphism (HVP) region at the 5′side of the JH region as well as the Cμ switch region (Sμ) are indicated (references 11 and 42). The solid bars represent the JH probe (ref. 41) and the Cμ probe (ref. 43).

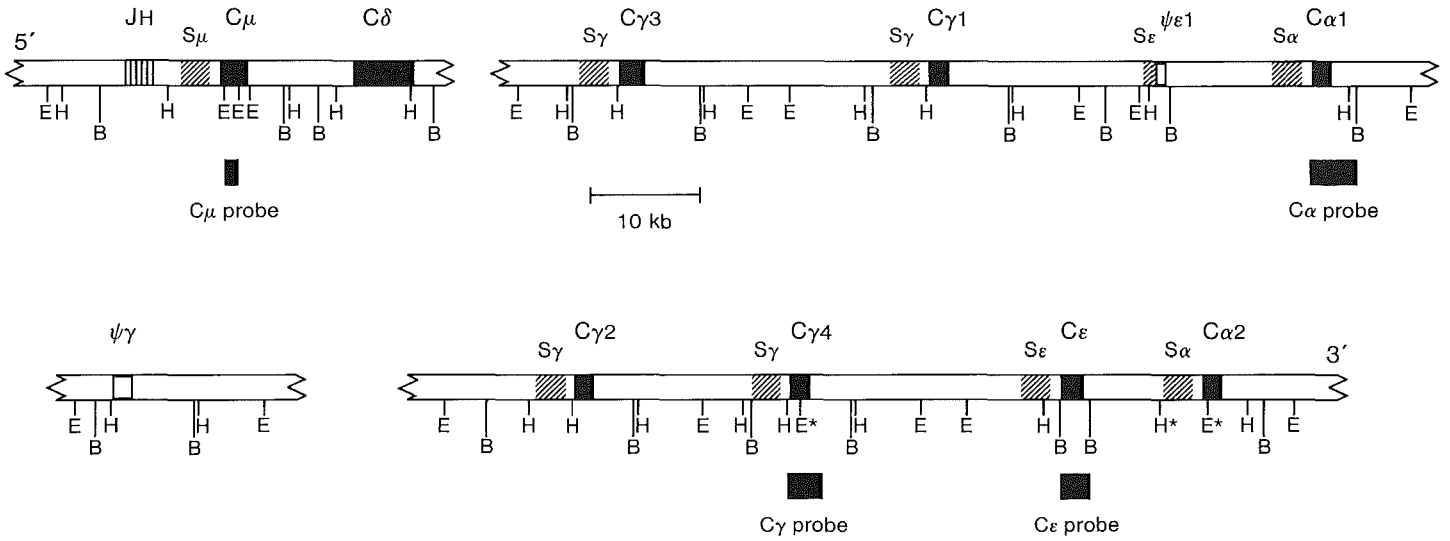


Figure 6. Organization of the CH regions of the human IgH genes (50-63). The locations of the relevant *Eco*RI (E), *Hin*dIII (H) and *Bam*HI (B) restriction sites are indicated. The polymorphic *Hin*dIII and *Eco*RI sites in the Cα2 gene, which are associated with the A2m(2) allotype, are indicated with an asterisk (16). Also the polymorphic *Eco*RI site in the Cγ4 gene is marked (17). The polymorphic *Bam*HI sites in the Cγ2, Cγ4 and ψγ genes are not given in this figure, but are described by Bech-Hansen and colleagues (14,15,17). The pseudo γ (ψγ) gene and pseudo ϵ1 (ψϵ1) gene are indicated as open boxes. The shaded areas indicate the approximate position of the CH switch regions (s)(16,42,54,60). The solid bars represent the Cμ probe (43), the Cγ probe (53), the Cα probe (56,60) and the Cϵ probe (60).

TABLE 2. Genomic probes for the detection of rearrangements and deletions in IgH genes.

DNA probe[a] (code name)	size of insert in kb (restriction enzymes)	restriction enzymes used for digestion of genomic DNA	approximate size of germline restriction fragments in kb (exon content of restriction fragment)
JH probe	2.5	*Bgl*II	3.9
(subclone of H24)[b]	(*Eco*RI-*Bgl*II)	*Bam*HI/*Hin*dIII	6.0
		*Hin*dIII[c]	9.5[c]
		*Eco*RI[c]	16.0[c]
		*Bam*HI	16.5
Cμ probe	1.2	*Bam*HI	16.5
(subclone of λC75)[d]	(*Eco*RI-*Eco*RI)	*Hin*dIII	11.5
Cγ probe	3.1	*Bam*HI	12.5 (Cγ1)
(subclone of λHG4.1)[d]	(*Hin*dIII-*Eco*RI)		13.5 or 25[e] (Cγ2)
			11.8 (Cγ3)
			9.0 or 9.4[e] (Cγ4)
			8.8 or 10.0[e] (ψγ)
		*Eco*RI	26.5 (Cγ1)
			23.5 (Cγ2)
			21.0 (Cγ3)
			20.0 (or 11.0 and 9.0)[e] (Cγ4)
			17.0 (ψγ)
		*Hin*dIII	7.8 (Cγ1, Cγ3 and ψγ)
			6.3 (Cγ4)
			6.0 (Cγ2)
Cα probe	4.3	*Bam*HI	17.0 (Cα1)
(subclone of Huα1)[f]	(*Xho*I-*Bam*HI)		16.0 (Cα2)
		*Eco*RI	25 (Cα1)
			30 (or 8.0)[g] (Cα2)
		*Hin*dIII	18.3 (Cα1)
			18.7 (or 8.0)[g] (Cα2)
		*Pst*I[h]	1.2 (Cα1)
			2.0 (Cα2)
Cε probe	2.6	*Bam*HI	2.6 (Cε)
(subclone of 38A)[f]	(*Bam*HI-*Bam*HI)		6.0 (ψε1)
			9.0 (ψε2)
		*Eco*RI	30 (or 22)[g] (Cε)
			25 (ψε1)
			(ψε2)
		*Hin*dIII	18.7 (or 10.7)[g] (Cε)
			18.3 (ψε1)
			8.5 (ψε2)

a. The various probes are presented in Figure 5 (JH and Cμ probes) and Figure 6 (CH probes).
b. Dr. T. Honjo (Kyoto Japan) kindly provided the JH probe (ref. 41).
c. The *Hin*dIII and *Eco*RI germline restriction fragments of the JH region may vary in size due to the presence of a hypervariable polymorphic region at the 5′ side of the JH region (ref. 11).
d. Dr. T.H. Rabbitts (Cambridge, UK) kindly provided the Cμ probe (ref. 43) and the Cγ probe (ref. 53).
e. *Bam*HI and *Eco*RI restriction fragment polymorphisms as described by Bech Hansen and colleagues (ref. 14,15 and 17).
f. Dr.Ph.Leder (Boston,MA) kindly provided the Cα probe (ref. 56 and 60) and the Cε probe (ref. 60).
g. *Eco*RI and *Hin*dIII restriction fragment polymorphisms; the presence of these restriction sites is associated with the A2m(2) allotype of the Cα2 gene (ref. 16).
h. The *Pst*I restriction sites are not indicated in Figure 6, but can be found in ref. 16.

figuration of the JH and Cμ gene region, the JH probe and the sizes of the germline restriction fragments are summarized in Figure 5 and Table 2 (11,41-44).

IgH class switches and the accompanying CH gene deletions can be studied with CH region probes (45-48). Although a Cγ3 specific probe, recognizing the Cγ3 hinge region, has been isolated (49), all other available Cγ, Cα and Cε probes cross-hybridize to CH region (pseudo) genes of the same IgH class (50-62). Particularly in case of Cγ probes this results in multiple bands, of which the germline patterns are complex due to various polymorphisms (see below and Table 2)(14-17). The investigation of IgH class switches has an additional complication, because an IgH class switch also occurs in the allelically excluded IgH gene in 75% of the IgG and IgA expressing B cell tumors (47). Such class switches are variable: i.e. 5′ of or 3′ of the CH region of the productive IgH gene allele (47). Nevertheless in many B cell malignancies it will be possible to study IgH class switches by the combined use of a JH probe and a CH probe (47,48). In case of restriction fragment polymorphisms, this study is facilitated by the availability of germline DNA from the same individual.

Germline *Bam*HI restriction fragments, which contain the complete JH region, span the Cμ gene and its switch region. No *Bam*HI restriction sites are located between the other CH regions and their accompanying switch regions (Figure 6)(42,43,50-63). Therefore JH gene segments and a switched CH gene are located on the same rearranged *Bam*HI restriction fragment. This can be demonstrated by sequential hybridization of the same *Bam*HI filter with the JH and the appropriate CH probe (47,48). Similarly, in *Eco*RI digests the JH gene segments and the switched CH genes are located on the same restriction fragment. However, most germline and rearranged CH gene containing *Eco*RI fragments are large (generally > 20 bands, of which the germline patterns are complex due to various polymorphisms (see below and Table 2)(14-17). The investigation of IgH class switches has an additional complication, because an IgH class switch also occurs in the allelically excluded IgH gene in 75% of the IgG and IgA expressing B cell tumors (47). Such class switches are variable: i.e. 5′ of, equal to, or 3′ of the CH region of the productive IgH gene allele (47). Nevertheless in many B cell malignancies it will be possible to study IgH class switches by the combined use of a JH probe and CH probe (47,48). In case of restriction fragment polymorphisms, this study is facilitated by the availability of germline DNA from the same individual.

Germline *Bam*HI restriction fragments, which contain the complete JH region, span the Cμ gene and its switch region. No *Bam*HI restriction sites are located between the other CH regions and their accompanying switch regions (Figure 6)(42,43,50-63). Therefore JH gene segments and a switched CH gene are located on the same rearranged *Bam*HI restriction fragment. This can be demonstrated by sequential hybridization of the same *Bam*HI filter with the JH and the appropriate CH probe (47,48). Similarly in *Eco*RI digests the JH gene segments

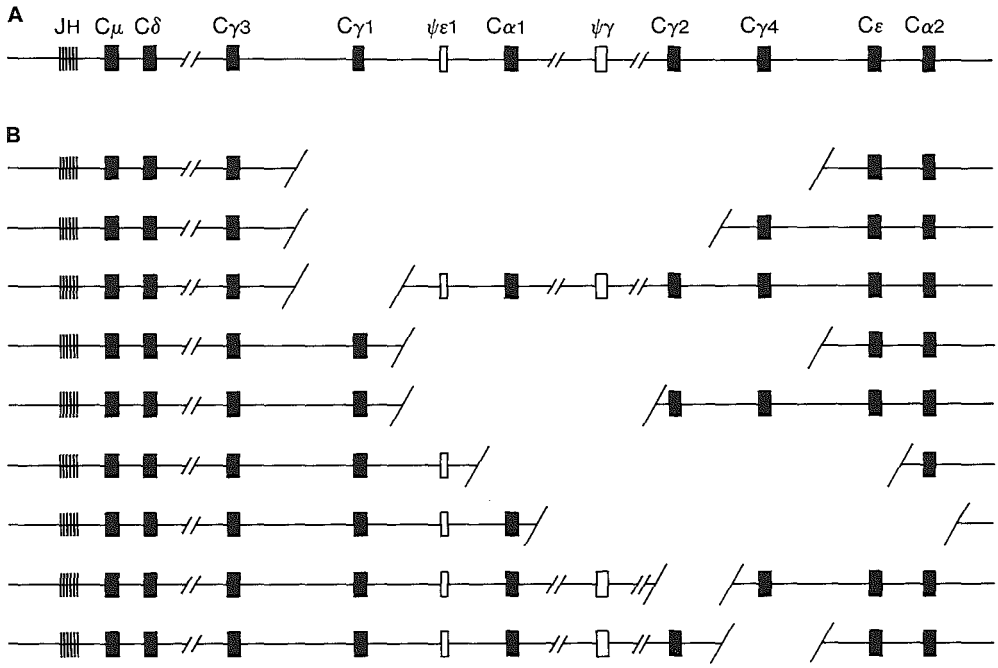


Figure 7. Inherited CH gene deletions. **A:** Germline organization of the human CH gene regions. **B:** Nine different types of inherited CH gene deletions, which all occur in the Cγ3-Cα2 gene cluster: Cγ1-Cγ4 deletion (64,65), Cγ1-Cγ2 deletion (66), Cγ1 deletion (66), ψε1-Cγ4 deletion (67,68), ψε1-ψγ deletion (65), Cα1-Cε deletion (67), ψγ-Cα2 deletion (70,71), Cγ2 deletion (70,71) and Cγ4 deletion (71).

and the switched CH genes are located on the same restriction fragment. However, most germline and rearranged CH gene containing *Eco*RI fragments are large (generally > 20 kb)(Table 2)(50,53,54,59,60).

Deletions of CH genes are detectable in *Bam*HI and *Eco*RI digests, and also *Hin*dIII and *Pst*I digests can give additional information (Figure 6 and Table 2).

It must be emphasized that deletions of one or more IgH-C genes may sometimes occur as an inherited aberration. So far, nine different types of inherited IgH-C gene deletions have been discovered: all these occur in the Cγ3-Cα2 gene cluster and do not involve the Cμ-Cδ gene segment (Figure 7)(64-71). It has been estimated that about 3% of individuals is heterozygous for these inherited IgH-C gene deletions (67,71).

Igκ genes

Rearrangements in the Igκ genes are detectable in *Bam*HI digests with a Cκ probe and/or a Jκ probe and in *Sac*I, *Hin*dIII and *Eco*RI digests with the Jκ probe (Figure 8; Table 3)(72,73). In most $Ig\lambda^+$ B cells or precursor B cells with rearranged Igλ genes the Cκ gene region or the whole Jκ-Cκ gene region is deleted on both alleles (74,75). These deletions are detectable with

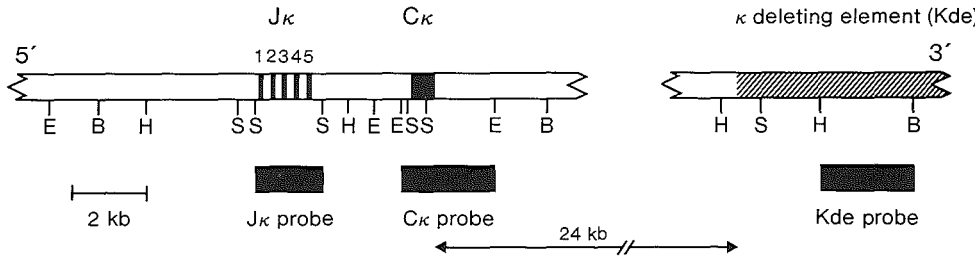


Figure 8. Organization of the J and C regions as well as the κ deleting element (Kde) region of the human Igκ genes (72,73,77,78). The location of the *Eco*RI (E), *Bam*HI (B), *Hin*dIII (H) and *Sac*I (S) restriction sites are indicated. The shaded area represents the Kde region. The solid bars represent the Jκ and Cκ probes (72,73) and the Kde probe (76,77).

the Kde probe, which recognizes a gene region (κ deleting element; Kde) that rearranges during the deletion process (76-78). The Kde probe may be important in cases where additional investigations must be performed on samples containing a small malignant $Sm\lambda^+$ B cell population amongst normal cells with germline Igκ genes. In such cases, deletions in the Igκ genes are likely to be detectable with the Kde probe, while the Jκ and/or Cκ probes may be insufficient, due to the presence of germline bands from the "contaminating" normal cells.

Igλ genes

Six Cλ genes, each preceded by a J gene segment, are located on a 40 kb DNA segment (79,80). Two of these Cλ genes are pseudogenes (ψλ4 and ψλ5), while four are functional (81). In addition to this well-established Igλ gene locus several other λ genes have been reported, including two pseudogenes and two potentially functional λ genes (82-84; Dr. B. Blomberg, personal communication).

In most studies possible rearrangements in the classical Igλ gene locus are investigated in *Eco*RI digests with a Cλ probe (Figure 9). In germline Igλ genes without polymorphic amplification of the λ genes, this results in three bands: 8.3 kb (Cλ2 and Cλ3), 14.0 kb (Cλ1) and 17.0 kb (ψλ4, ψλ5 and Cλ6)(Figure 9 and Table 3). In case of polymorphic amplifications of the Cλ2-Cλ3 gene segment, other bands will be detectable (Figures 9 and 10 and Table 3)(12). For a correct interpretation of the banding pattern, it is important to know whether a particular band is due to the well-established λ polymorphism or to rearrangement. For this purpose a probe which detects the polymorphic λ bands (λ-IVS probe) and, if available, control DNA from the same individual should be used (Figure 10)(12). In *Eco*RI/*Hin*dIII digests the occurrence of polymorphic amplification of the Cλ2-Cλ3 gene segment is detected as a 5.4 kb band, irrespective of the type of polymorphism (Figure 11)(12). This can facilitate the analysis of Igλ genes, as demonstrated in 22 Igλ-expressing B cell leukemias. *Eco*RI digests in combination with the Cλ probe detected rearrangements in 20 of the 22 leukemias, while *Eco*RI/*Hin*dIII double digests allowed detection of Igλ gene rearrangements in all 22 $Ig\lambda^+$ leukemias (W.M. Comans-Bitter, unpublished results).

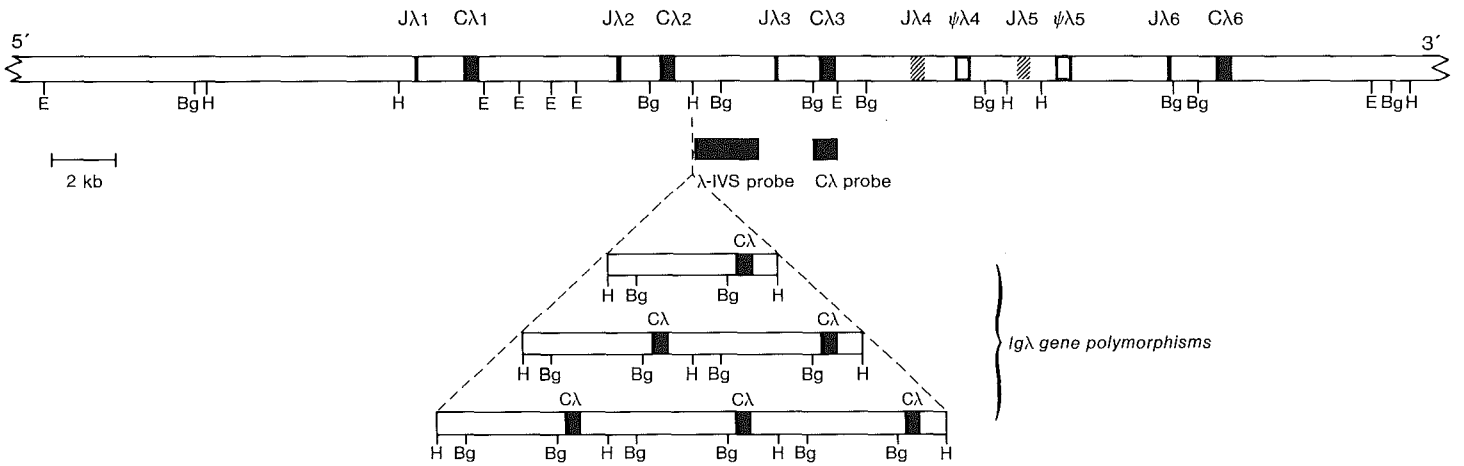


Figure 9. Organization of the J and C regions of the human Igλ genes (12,79-81). The *Eco*RI (E), *Bgl*II (Bg) and *Hind*III (H) restriction sites are indicated. The ψλ4 and ψλ5 genes are presented as open boxes and the shaded areas indicate the approximate positions of the Jλ4 and Jλ5 genes (81). The polymorphic amplifications of the Cλ2-Cλ3 gene segments are presented in the lower part of the figure (12). The solid bars represent the Cλ probe (79) and λ-IVS probe (12).

TABLE 3. Genomic probes for the detection of rearrangements and deletions in IgL genes.

DNA probe[a] (code name)	size of insert in kb (restriction enzymes)	restriction enzymes used for digestion of genomic DNA	approximate size of germline restriction fragments in kb (exon content of rectriction fragment)	
Igκ genes				
Jκ probe (from Huκ)[b]	1.8 (*Sac*I-*Sac*I)	*Sac*I	1.8	
		*Hin*dIII	5.4	
		*Eco*RI	8.7	
		*Bam*HI	12.0	
Cκ probe (from Huκ)[b]	2.5 (*Eco*RI-*Eco*RI)	*Bam*HI	12.0	
Kde probe (Kde-*Nalm*-11.5)[c]	2.5 (*Hin*dIII-*Bam*HI)	*Bam*HI	14.0 (and 2.5)[d]	
Igλ genes				
Cλ probe (from Huλ C2, C3)[b]	0.8 kb (*Bgl*II-*Eco*RI)	*Eco*RI	14.0	(Cλ1)
			8.3	(Cλ2 and Cλ3)
			17.0	(ψλ4, ψλ5 and Cλ6)
				(13.7, 19.1 or 24.5 in case of Cλ polymorphisms)[e]
		*Hin*dIII/*Eco*RI	2.7	(Cλ1)
			3.7	(Cλ2)
			4.6	(Cλ3)
			5.4	(ψλ4)
			10.5	(ψλ5 and Cλ6)
				(5.4 in case of Cλ polymorphisms)[e]
λ-IVS probe (from Huλ C2,C3)[b]	2.1 (*Hin*dIII-*Bam*HI)	*Eco*RI	8.3	(13.7,19.1 or 24.5 in case of Cλ polymorphisms)[e]
		*Hin*dIII/*Eco*RI	3.7	(λ2-IVS)
			4.6	(λ3-IVS)
				(5.4 in case of Cλ polymorphisms)[e]

a. The various probes are presented in Figure 8 (Jκ, Cκ and Kde probes) and Figure 9 (Cλ and λ-IVS probe).
b. Dr. Ph. Leder (Boston, MA) kindly provided the Jκ probe and Cκ probe (ref. 72,73) as well as the Cλ probe (ref. 79) and the λ-IVS probe (ref. 12).
c. Dr. S. Korsmeyer (Saint Louis, MO) kindly provided the Kde probe (ref. 76,77).
d. The 2.5 kb *Hin*dIII-*Bam*HI Kde probe not only recognizes the κ deleting element which rearranges in order to delete Cκ or Jκ-Cκ, but also cross-hybridizes to sequences on the long arm of chromosome 2 (2q11). In a *Bam*HI digest these crosshybridizing sequences are located on a 2.5 kb fragment (76,77).
e. Within the Igλ gene locus a variable amplification of the Cλ2-Cλ3 gene segment may occur. This genetic polymorphism results in 13.7 kb, 19.1 kb or 24.5 kb bands in case of *Eco*RI digests as detected by a Cλ probe or λ-IVS probe (Figure 10) or in a 5.4 kb band in case of *Hin*dIII/*Eco*RI digests as detected by a Cλ probe or λ-IVS probe (Figure 11).

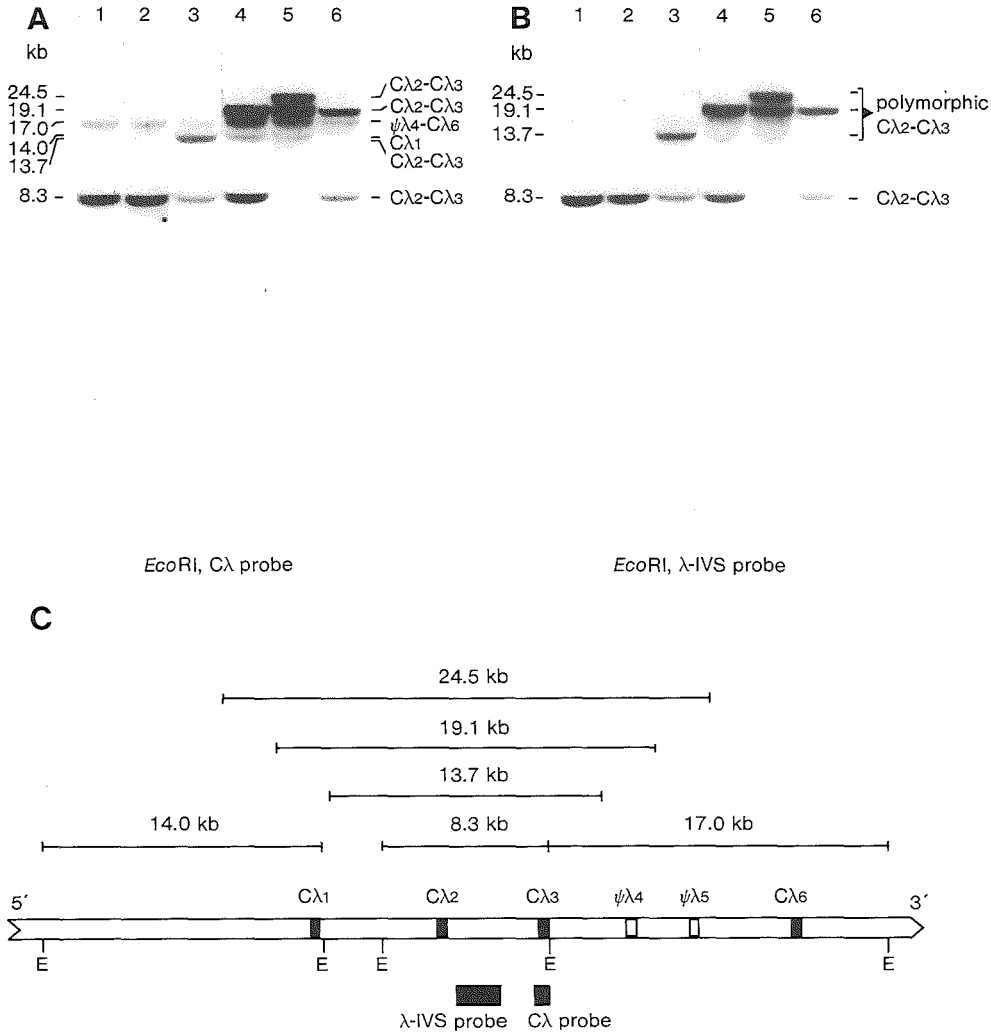


Figure 10. Polymorphic amplification of the Cλ2-Cλ3 gene segment as detected in *Eco*RI digests. **A**: *Eco*RI digested DNA samples with germline Igλ genes from different individuals were size fractionated, blotted to a nylon membrane and hybridized with a ^{32}P-labeled Cλ probe. In case of germline Igλ genes without polymorphic amplification of the Cλ2-Cλ3 gene segment, three germline bands are present: 8.3 kb (Cλ2-Cλ3), 14.0 kb (Cλ1) and 17.0 kb (ψλ4-ψλ5-Cλ6). If the polymorphic amplification is present, also other bands are detected (13.7 kb, 19.1 kb and/or 24.5 kb), while the 8.3 kb band is reduced in intensity (polymorphism of one allele) or absent (polymorphism of both alleles). **B**: The *Eco*RI filter is now hybridized with the λ-IVS probe, which detects the Cλ2-Cλ3 gene segment. In case of the polymorphic amplification one or two large bands are detectable, while in absence of the polymorphism only the 8.3 kb band is detected. **C**: Schematic diagram of the polymorphic amplification of the Cλ2-Cλ3 gene segment. The *Eco*RI restriction sites and the relevant restriction fragments as well as the Cλ probe and the λ-IVS probe are indicated (c.f. reference 12).

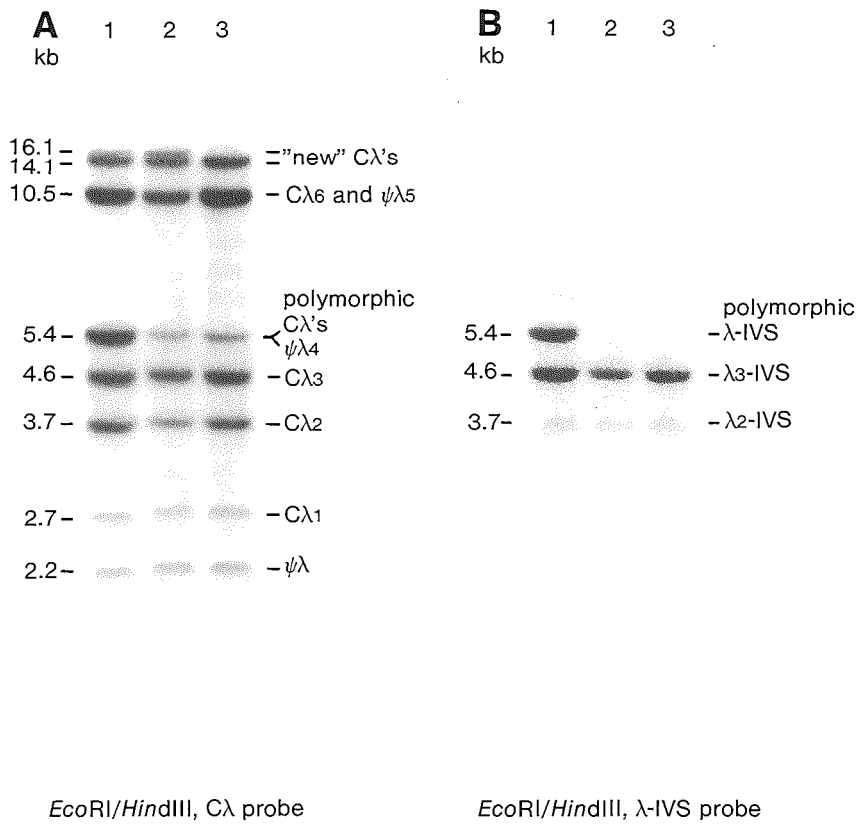


Figure 11. Polymorphic amplification of the Cλ2-Cλ3 gene segment as detected in *Eco*RI/*Hin*dIII digests. **A**: DNA samples with germline Igλ genes from three different individuals were digested with a combination of *Eco*RI and *Hin*dIII, size fractionated, blotted to a nylon membrane and hybridized with a ^{32}P-labeled Cλ probe. The Cλ1, Cλ2, Cλ3, ψλ4 and ψλ5-Cλ6 gene segments are located on separate restriction fragments of 2.7, 3.7, 4.6, 5.4 and 10.5 kb, respectively. In case of polymorphic amplification of the Cλ2-Cλ3 gene segment (lane 1), the 5.4 kb band is stronger than in absence of the polymorphism (lanes 2 and 3). The 2.2, 14.1 and 16.1 kb bands contain Cλ genes, which are located outside the "classical" Igλ gene locus (82-84; Dr. B. Blomberg, personal communication). **B**: When the *Eco*RI/*Hin*dIII filter is hybridized with the λ-IVS probe, the strong 5.4 kb band only remains in case of the polymorphism (lane 1). In *Eco*RI/*Hin*dIII digests all different types of polymorphic amplifications of the Cλ2-Cλ3 gene segment result in the same 5.4 kb band (c.f. Figure 9).

In both *Eco*RI and *Eco*RI/*Hin*dIII digests faint germline bands are detectable with a Cλ probe. Several of these bands have been demonstrated to represent new λ (pseudo) genes (82-84; Dr. B. Blomberg, personal communication).

Despite the complexity of the Igλ genes, it will generally be possible to detect their rearrangements in large malignant Igλ$^+$ B cell populations. However, if the clonal Igλ$^+$ B cell population is small, the rearranged Igλ gene bands may be difficult to detect. In such case, the Kde probe can be useful for the detection of deletions in Igκ genes, which occur in most Igλ$^+$ cells (see above).

TABLE 4. Genomic probes for the detection of rearrangements and deletions in the TcR-β, TcR-γ and TcR-δ genes.

DNA probe[a] (code name)	size of insert in kb (restriction enzymes)	restriction enzymes used for digestion of genomic DNA	approximate size of germline restriction fragments in kb (exon content of restriction fragment)	
TcR-β genes				
Jβ1 probe (Jβ1 subclone of Cβ50)[b]	4.0 (*Hin*dIII-*Hin*dIII)	*Eco*RI	10.5	(Jβ1 and Cβ1)
		*Hin*dIII	4.0	(Jβ1)
Jβ2 probe (Jβ2 subclone of Cβ47)[b]	1.9 (*Pvu*II-*Pvu*II)	*Eco*RI	4.1 (7.9)[c]	(Jβ2)
		*Hin*dIII	7.5	(Jβ2 and Cβ2)
Cβ probe (5'Cβ2 subclone of Cβ47)[b]	1.1 (*Eco*RI-*Hin*dIII)	*Bam*HI	22.5	(β1 and β2 genes)
		*Eco*RI	10.5	(Jβ1 and Cβ1)
			3.8 (7.9)[c]	(Cβ2)
		*Hin*dIII	3.5	(Cβ1)
			7.5	(Jβ2 and Cβ2)
TcR-γ genes				
Jγ2.1 probe (subclone of pγC1C2)[d]	0.2 (*Hin*dIII-*Eco*RI)	*Eco*RI	2.3	(Jγ1.1)
			1.2	(Jγ2.1)
Jγ1.2 probe (subclone of λ33)[d]	1.2 (*Hin*dIII-*Eco*RI)	*Eco*RI	2.5	(Jγ1.2)
Jγ1.3 probe (subclone of λ33)[d]	0.8 (*Hin*dIII-*Eco*RI)	*Eco*RI	1.8	(Jγ1.3)
			3.3 (or 1.8)[e]	(Jγ2.3)
		*Kpn*I[f]	9.0	(all Jγ1 genes)[f]
			16.0	(all Jγ2 genes)[f]
Cγ probe (Cγ1 clone)[b]	0.4 (*Bam*HI-*Bam*HI)	*Eco*RI[g]	5.0	(Cγ1)
			6.8	(Cγ2)
		*Bam*HI	20.0	(γ1 genes)
			12.5	(γ2 genes)
TcR-δ genes				
Jδ1 probe (pJδS16)[h]	1.5 (*Sac*I-*Sac*I)	*Eco*RI	6.0 and 5.0	(Jδ1)
		*Hin*dIII	6.0	(Jδ1)
		*Kpn*I	16.0	(Jδ1)
		*Bam*HI	17.5	(Jδ1 and Jδ2)
Jδ2 probe (R21XH)[h]	2.3 (*Eco*RI-*Hin*dIII)	*Eco*RI	5.7	(Jδ2)
		*Hin*dIII	3.5	(Jδ2)
		*Kpn*I/*Bam*HI	6.4	(Jδ2)
		*Bam*HI	17.5	(Jδ1 and Jδ2)
		*Kpn*I	17.0	(Jδ2-Vδ3)
Cδ probe (pCTCRδ2c)[i]	0.7[i] (*Eco*RI-*Eco*RI)	*Bam*HI/*Sac*I	8.0	(Jδ3 and Cδ)
		*Bam*HI	14.0	(Jδ3-Vδ3)
		*Kpn*I	17.0	(Jδ2-Vδ3)
		*Bgl*II	4.5 and 3.1	(Cδ and Vδ3)

a. The various probes are presented in Figure 12 (TcR-β probes), Figure 13 (TcR-γ probes) and Figure 15 (TcR-δ probes).
b. Dr. J. Seidman (Boston, MA) kindly provided the Jβ1 probe (ref. 86,89), the Jβ2 probe (ref. 86,89), the Cβ probe (ref. 86) and the Cγ probe (ref. 92,95).
c. The *Eco*RI site between the Jβ2 gene and Cβ2 gene is sometimes partly resistant to digestion. This results in a 7.9 kb band which hybridizes to both the Jβ2 probe and the Cβ probe.
d. Dr. T. Quertermous (Boston, MA) kindly provided the Jγ2.1 probe, Jγ1.2 probe and the Jγ1.3 probe (ref. 92,95).
e. In some individuals a polymorphic *Eco*RI site is present at the 5′ side of the Jγ2.3 gene segment, which results in a Jγ2.3 restriction fragment of 1.8 kb (Figure 14)(20).
f. Each rearrangement to any of the Jγ1 or Jγ2 gene segments can be identified with the Jγ1.3 probe, when the DNA is digested with *Kpn*I (ref. 93).
g. Hybridization of the Cγ probe to *Eco*RI digested DNA is useful for the detection of Cγ1 gene deletions.
h. Dr. T.H. Rabbitts (Cambridge, UK) kindly provided the Jδ1 probe and the Jδ2 probe (ref. 36,104).
i. Dr. P. van den Elsen (Leiden, The Netherlands) kindly provided the Cδ probe. This is a cDNA probe, which only contains human Cδ sequences (101).

T CELL RECEPTOR PROBES

TcR-β genes

Rearrangements in the TcR-β genes are generally detectable by the combined use of a Jβ2 probe and a Cβ probe in *Eco*RI and *Bam*HI digests. Also the Jβ1 probe and *Hin*dIII digests can give additional information (Figure 12 and Table 4)(85-89).

The Cβ probe recognizes both Cβ1 and Cβ2 sequences (90), while Jβ probes do not cross-hybridize. Rearrangements to the Jβ1 region are detectable with the Cβ probe in *Eco*RI digests or with the Jβ1 probe in *Hin*dIII digests. Rearrangements to the Jβ2 region can be detected with the Jβ2 probe in *Eco*RI digests or with the Cβ probe in *Hin*dIII digests.

Importantly, the *Eco*RI and *Hin*dIII restriction sites in the Cβ2 region may be partly resistant to digestion (91). Such an underdigestion leads to an extra germline band, not to be mistaken for a rearranged one (Figure 12 and Table 4).

TcR-γ probes

All rearrangements in the TcR-γ genes are detectable with the Cγ probe in *Bam*HI digests (19,92) or with the Jγ1.3 probe in *Kpn*I digests (93). This is based on the capacity of the Cγ

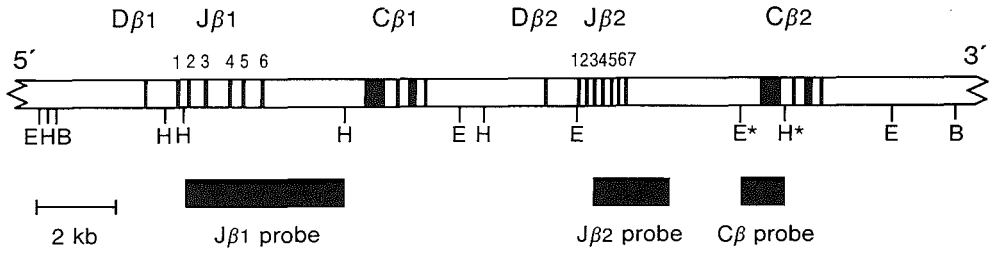


Figure 12. Organization of the Dβ, Jβ and Cβ regions of the human TcR-β genes (85-89). The locations of the relevant *Eco*RI (E), *Hin*dIII (H) and *Bam*HI (B) restriction sites are indicated. The partly resistant *Eco*RI and *Hin*dIII sites in the Cβ2 region are indicated with an asterisk (91). The solid bars represent the Jβ1 probe, Jβ2 probe and Cβ probe (86,89).

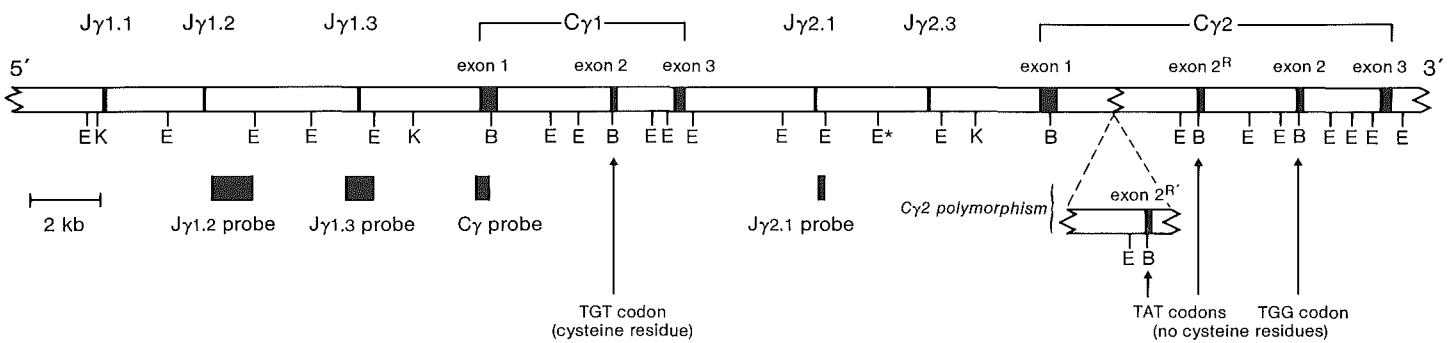


Figure 13. Organization of the J and C regions of the human TcR-γ genes (13,19,21,92-96). The locations of the relevant *Eco*RI (E), *Kpn*I (K) and *Bam*HI (B) restriction sites are indicated (19,92,93). The solid bars represent the TcR-γ probes (92). The polymorphic *Eco*RI site at the 5′side of the Cγ2 gene is marked with an asterisk (20). In the second exon of the Cγ1 gene the TGT codon codes for the cysteine residue which is presumably involved in the interchain disulfide bond, while in the duplicated or triplicated second exon (exon $2^{R'}$, exon 2^{R} and exon 2) this codon has been changed into TAT or TGG (13,19,21,97).

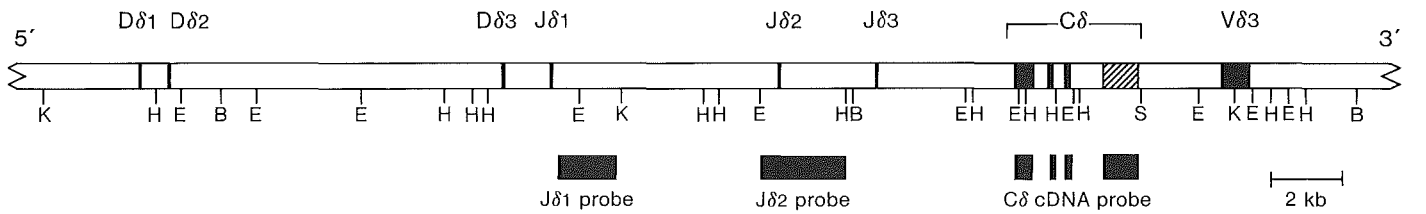


Figure 15. Organization of the D, J and C regions of the human TcR-δ genes (24,36,101,104-107). The locations of the relevant *Kpn*I (K), *Hind*III (H), *Eco*RI (E) and *Bam*HI (B) restriction sites are indicated. Only one *Sac*I (S) site is given; further information on the location of *Sac*I sites is given in reference 36. The Vδ3 gene segment is located within 3 kb at the 3′side of the Cδ region (107). The solid bars represent the Jδ1 probe (36,104), the Jδ2 probe (36) and the Cδ probe (101). The latter probe is a cDNA probe, which only contains Cδ sequences.

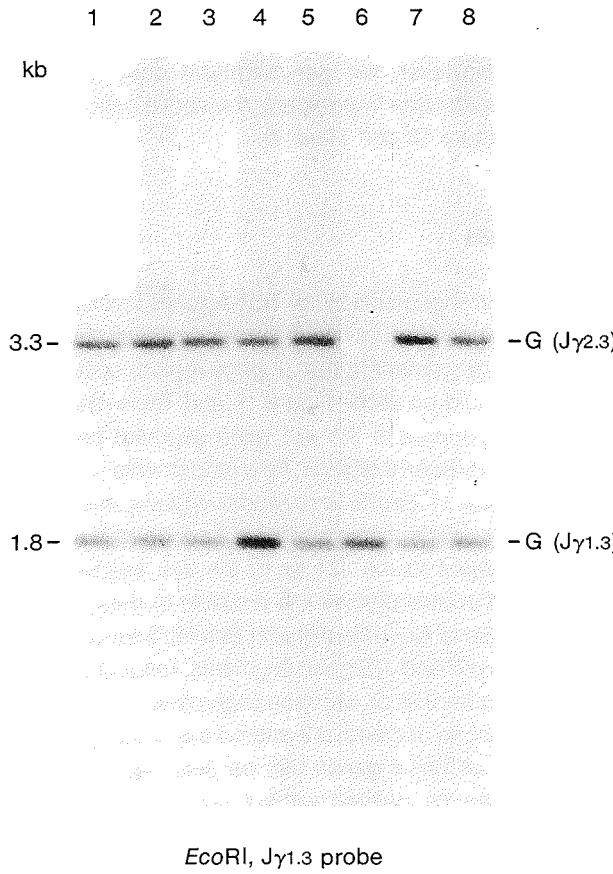


Figure 14. Southern blot analysis of the TcR-γ genes, illustrating the polymorphic presence of an *Eco*RI restriction site at the 5′ side of the Jγ2.3 gene segment (20). *Eco*RI digested DNA samples with germline TcR-γ genes from different individuals were size fractionated, blotted to a nylon membrane and hybridized to the Jγ1.3 probe (Figure 13). In most individuals the Jγ1.3 and Jγ2.3 gene segments reside on restriction fragments with a different size (1.8 kb and 3.3 kb, respectively). In some individuals a polymorphic *Eco*RI site is present at the 5′ side of the Jγ2.3 gene segment, which results in Jγ1.3 and Jγ2.3 restriction fragments of identical size (20).

probe to hybridize to both Cγ1 and Cγ2 sequences and of the Jγ1.3 probe to recognize both Jγ1.3 gene and Jγ2.3 gene segments (92). A detailed analysis of the TcR-γ gene rearrangements can be performed with the Jγ2.1, Jγ1.2, and Jγ1.3 probes in *Eco*RI digests (92). Like the Jγ1.3 probe, the Jγ2.1 probe cross-hybridizes to its equivalent in the TcRγ1 locus, i.e. the Jγ1.1 gene segment (92). Detailed information about the configuration of the TcR-γ genes and the various TcR-γ probes is summarized in Figure 13 and Table 4 (13,19,21,92-96). Rearrangements to the Jγ2 gene segments will delete the TcR-γ1 gene locus. This deletion is easily detectable with the Cγ probe and the Jγ1.3 probe in *Eco*RI digests, the Cγ probe in *Bam*HI digests and the Jγ1.3 probe in *Kpn*I digests (Figure 13).

Two types of polymorphisms in the TcR-γ genes have been described. One concerns the occurrence of a polymorphic *Eco*RI site at the 5′ side of the Jγ2.3 gene segment (Figure 13)(20). In most individuals this site is absent, which results in a germline Jγ2.3 restriction fragment of 3.3 kb. If this site is present, the size of the *Eco*RI restriction fragments of both the Jγ1.3 and the Jγ2.3 gene segments is 1.8 kb (Figures 13 and 14). Therefore, the presence of the polymorphic *Eco*RI site should not be misinterpreted as a deletion of the Jγ2.3 gene

segment (Figure 14). The second type of polymorphism concerns the second exon of the Cγ2 gene region, which is duplicated in two-third of alleles and triplicated in one-third of alleles (13,21,97). However, this polymorphism does not influence the sizes of the germline and rearranged restriction fragments in the above described combinations of probes and restriction enzymes (Figure 13 and Table 4).

TcR-δ probes

Most rearrangements in the TcR-δ genes involve Jδ gene segments (98-101), although V-D and D-D joining with germline Jδ genes has been described as well (102,103). For the detection of each rearrangement in the Jδ gene region, several combinations of probes and restriction enzymes should be used (Figure 15 and Table 4)(24,36,101,104-107).

Rearrangements to the Jδ1 gene segment are detectable with the Jδ1 probe in *Eco*RI, *Hin*dIII, *Kpn*I or *Bam*HI digests. Rearrangements to the Jδ2 gene segment can be detected with the Jδ2 probe in *Eco*RI and *Hin*dIII digests and, if necessary, they can be confirmed in *Kpn*I/*Bam*HI double digests. The Jδ2 probe in a *Bam*HI digest cannot discriminate between a rearrangement to the Jδ1 or to the Jδ2 segment (Figure 15). By using the Cδ probe in *Bam*HI/*Sac*I double digests it is possible to detect rearrangements to the Jδ3 gene segment (101). The use of *Kpn*I/*Bam*HI and *Bam*HI/*Sac*I double digests for the detection of rearrangements to the Jδ2 and Jδ3 gene segments, respectively, prevents that Vδ3 gene rearrangements are mistaken for Jδ2 or Jδ3 rearrangements. The Vδ3 gene is located on the same germline *Kpn*I fragment as Jδ2 and Jδ3 and on the same germline *Bam*HI fragment as Jδ3 (Figure 15).

Deletion of TcR-δ genes can be detected with the Cδ probe in any digest. TcR-δ gene segments can be deleted without rearrangement of the TcR-α genes (98,108). Such an intermediate stage of rearrangement in the TcR-α/δ gene cluster can be determined by the recently described δRec and ψJα probes (98,108).

LIMITATIONS AND PITFALLS OF SOUTHERN BLOT ANALYSIS OF Ig AND TcR GENES

Since Southern blot analysis of Ig and TcR genes, has several limitations and pitfalls (Table 5) the results should always be interpreted with utmost caution.

Polymorphisms of Ig and TcR genes

Several polymorphisms have been shown to occur in the Ig and TcR genes. Some of these involve the target sequences of restriction enzymes, resulting in restriction fragment length polymorphisms (RFLP). Examples are:

- Polymorphic *Bam*HI restriction sites around the IgH-Cγ gene regions (14,15,17).
- Polymorphic *Eco*RI site in the IgH-Cγ4 gene segment (Figure 6)(17).
- Polymorphic *Hin*dIII and *Eco*RI sites in the Cα2 gene region. The presence of these restriction sites is associated with the A2m(2) allotype, which occurs at a low frequency

in Caucasians (1-2%), and at a higher frequency in Africans and Asians (50-80%)(16).
- Polymorphic *Bgl*II site at the 5´ side of the TcR-Cβ2 gene exons (18).
- Polymorphic *Eco*RI and *Hin*dIII sites at the 5´ side of the TcR-Jγ2.3 gene segment (Figures 13 and 14)(19,20,94).

A second type of polymorphism results from insertion or deletion of non-coding DNA segments and is designated hypervariable (or length) polymorphism (HVP). Such a HVP is found at the 5´ side of the IgH-J gene region (Figure 5)(11).

Finally, polymorphic amplifications or deletions of genes or gene segments are reported. These are:

- Inherited deletion of one or more IgH constant genes, which all occur in the Cγ3-Cα2 gene cluster (Figure 7)(64-71). About 1 - 3% of individuals is heterozygous for such a deletion (67,71).
- Polymorphic duplication of the IgH-Cγ2 gene. The frequency of this haplotype is estimated to be 0.043 (17).
- Polymorphic amplification of the Cλ2-Cλ3 gene segment in the Igλ genes (Figures 9-11). The frequencies of allelic duplication, triplication and quadruplication of this gene segment are estimated to be 0.05, 0.19 and 0.01, respectively (12).
- Polymorphic amplification of the second exon of the Cγ2 gene segment of the TcR-γ genes. In two-third of the TcR-γ gene alleles this second exon is duplicated, while in the remaining one-third this exon is triplicated (Figure 13)(13,19,21).

The above polymorphisms are the most frequently described, but as yet unreported polymorphisms may be found during diagnostic analysis of the Ig and TcR genes. It is therefore advisable to use germline control DNA and tumor DNA from the same patient.

TABLE 5. Limitations and pitfalls of the Southern blot analysis of Ig and TcR gene rearrangements.

1. Polymorphisms in Ig and TcR genes:
   - restriction site polymorphism (RFLP)
   - hypervariable (or length) polymorphism (HVP)
   - polymorphic amplification of genes or gene segments
2. Underdigestion of DNA.
3. Co-migration of a rearranged band with a germline band.
4. Limited combinatorial diversity of the TcR-γ and TcR-δ genes.
5. Multiple rearranged bands:
   - oligoclonality
   - chromosomal aberrations
6. Detection limit of the Southern blot technique (1-5%).

Underdigestion of DNA

Restriction enzyme digestions should always be checked for completeness by running parallel incubations with λ-DNA and/or plasmid-DNA (see above). Despite these controls, some digestions may still be incomplete. Especially the *Eco*RI site between the Jβ2 and Cβ2 gene

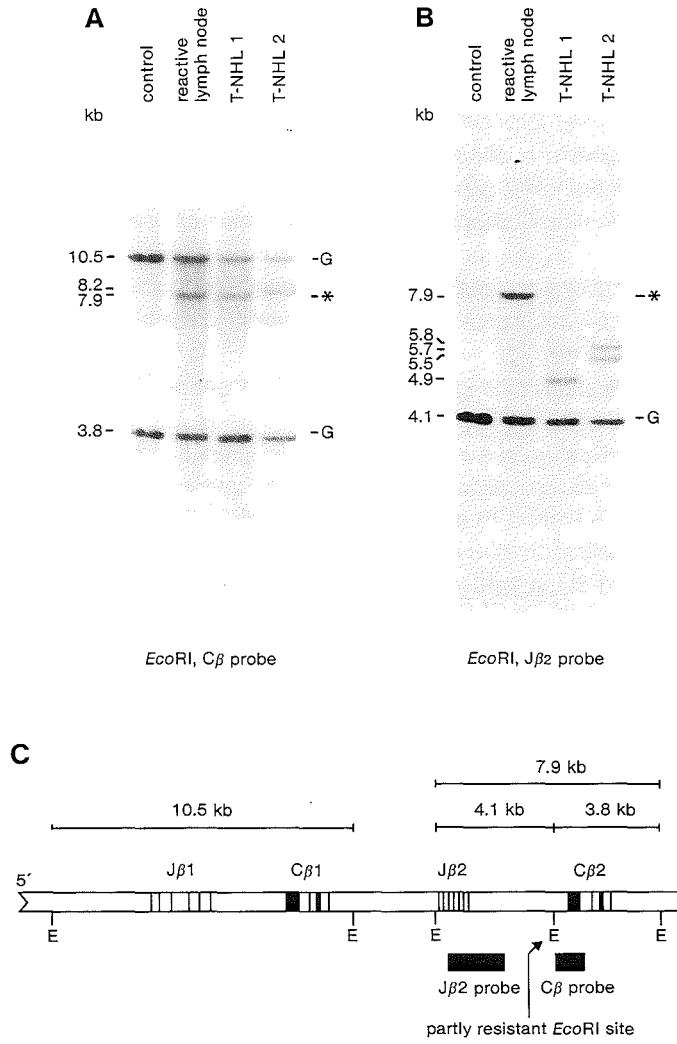


Figure 16. The *Eco*RI site between the Jβ2 and Cβ2 gene segments of the TcR-β genes is partly resistant to digestion (91,109). **A**: A germline control DNA sample, a DNA sample from a reactive lymph node as well as DNA samples from two lymph nodes with a T cell non-Hodgkin lymphoma (T-NHL) were digested with *Eco*RI, size separated and blotted to a nylon membrane. The membrane was hybridized with the ^{32}P-labeled Cβ probe. In the lanes of the reactive lymph node as well as in the lane of T-NHL-1 a 7.9 kb band was visible, while in the lane of T-NHL-2 a slightly larger band (~ 8.2 kb) was detected. **B**: The *Eco*RI membrane was also hybridized with the ^{32}P-labeled Jβ2 probe. Then the 7.9 kb was only present in the lane of the reactive lymph node, while in the two T-NHL lanes two rearranged bands were found. This illustrates that on the one hand a 7.9 kb band in an *Eco*RI digest using the Cβ probe may be caused by underdigestion (91), but that on the other hand such a 7.9 kb band may be caused by a rearrangement. **C**: Schematic diagram of the TcR-β genes. The locations of the *Eco*RI sites (E) as well as the Jβ2 probe and the Cβ probe are given. Also the position of the partly resistant *Eco*RI site and the sizes of the various germline bands are indicated.

segments of the TcR-β genes may appear to be partly resistant to digestion (Figure 12)(91,109). This leads to an additional "germline" band of 7.9 kb, which contains both Jβ2 and Cβ2 gene sequences. In completely digested DNA these gene segments are located on separate *Eco*RI fragments of 4.1 kb and 3.8 kb, respectively. The 7.9 kb band may be mistaken for a rearranged band or a polymorphism (110-115). Such a misinterpretation can be prevented by sequential hybridizations of the *Eco*RI membranes with the Jβ2 probe and Cβ probe (91). Both probes will hybridize to the 7.9 kb band, if this band is indeed due to underdigestion of the DNA. The importance of this approach is illustrated in Figure 16.

Also the *Hin*dIII site in the Cβ2 region of the TcR-β genes has been reported to be sometimes partly resistant to digestion (91).

Co-migration of a rearranged band with a germline band

In Southern blot analysis, discrimination between the presence of germline and rearranged Ig or TcR genes is based upon differences in size of the germline and rearranged bands. However, sometimes a rearranged band and a germline band may appear to have similar sizes, resulting in co-migration of the bands (Figures 16 and 17). There is little chance of co-migration, when the germline and rearranged bands are small (< 10 kb), but the likelihood of this event increases progressively with the size of the bands. For instance TcR-β gene rearrangements are often difficult to evaluate when studied in *Bam*HI digests using a Cβ probe.

The risk of misinterpretations due to co-migration of rearranged and germline bands can be eliminated, when at least two or even three different restriction enzyme digests are used for each probe. In addition, restriction enzymes which do not produce large germline bands should be preferred.

In our laboratory we generally use the JH probe in combination with a *Bgl*II digest and a *Bam*HI/*Hin*dIII digest for IgH gene analysis; if necessary, also *Bam*HI or *Eco*RI are used. The Jβ2 and Cβ probe in combination with *Eco*RI digests and *Bam*HI digests are used for studying TcR-β genes; if necessary, also the Jβ1 probe in combination with *Hin*dIII digests is used.

Limited combinatorial diversity of TcR-γ and TcR-δ genes

Discrimination between polyclonal and monoclonal lymphoid cell populations is possible because in a polyclonal lymphoproliferation many different Ig or TcR gene rearrangements are present, while the Ig or TcR gene rearrangements of a monoclonal lymphoproliferation are identical (Figure 1). This is the case for IgH, Igκ and Igλ genes as well as for TcR-α and TcR-β genes. However, the combinatorial repertoire of the TcR-γ genes and TcR-δ genes is limited, implying that polyclonal rearrangements of these genes will result in a restricted number of rearranged bands (20,92,93,95,96,101,106,107,116-120).

The combinatorial repertoire of the TcR-γ genes is determined by fifteen Vγ genes (eight functional V genes and seven pseudo V genes) and five Jγ genes (20,92,95,96,118). The TcR-δ genes consist of at least six Vδ genes, three Dδ genes and three Jδ genes (106,107,119,120). Since a large part of the TcR-$\gamma\delta^+$ blood T lymphocytes use Vγ9 and Jγ1.2 in combination with

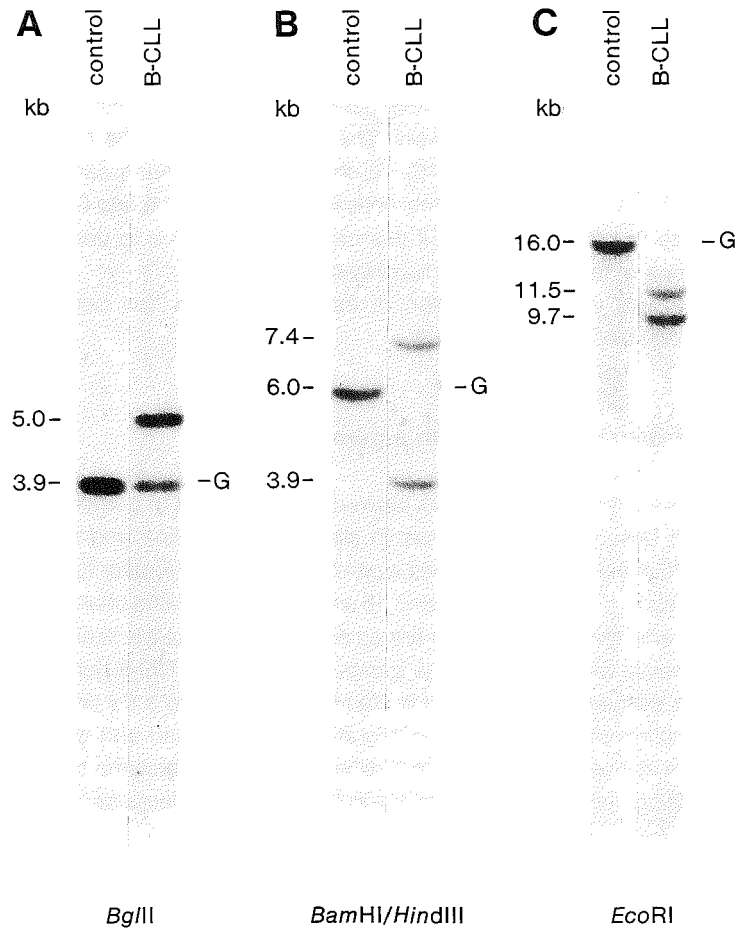


Figure 17. Southern blot analysis of the IgH genes of a B cell chronic lymphocytic leukemia (B-CLL). DNA from a control cell sample and DNA from a B-CLL sample were digested with *Bgl*II **(A)** and *Bam*HI/*Hin*dIII **(B)**, size-separated and blotted to a nylon membrane, which was hybridized with the ^{32}P-labeled JH probe. Although two rearranged bands were detectable in the *Bam*HI/*Hin*dIII digest, only one rearranged band was present in the *Bgl*II digest. This suggests that one rearranged band comigrated with the germline band. This interpretation was supported by the finding that also in an *Eco*RI digest **(C)** two rearranged bands were seen.

Vδ2 and Jδ1 (121,122), it can be assumed that the available TcR-γ and TcR-δ gene segments are not used randomly in normal TcR-γδ$^+$ cells. However, TcR-γ gene rearrangements in normal TcR-αβ$^+$ T lymphocytes as well as most T cell malignancies usually include a random use of one of the eight functional Vγ genes together with the Jγ2.3 or the Jγ1.3 gene segment (101,109,123-126). This results in a restricted number of rearranged bands as illustrated in Table 6 and Figure 18 (93,95,116,117). The background of rearranged TcR-γ gene bands from normal blood T lymphocytes hampers the detection of a clonal T cell population. From Figure 18 it can be concluded that a clonal T cell population should take up at least 45% of the cell sample analyzed to be detectable by TcR-γ gene analysis.

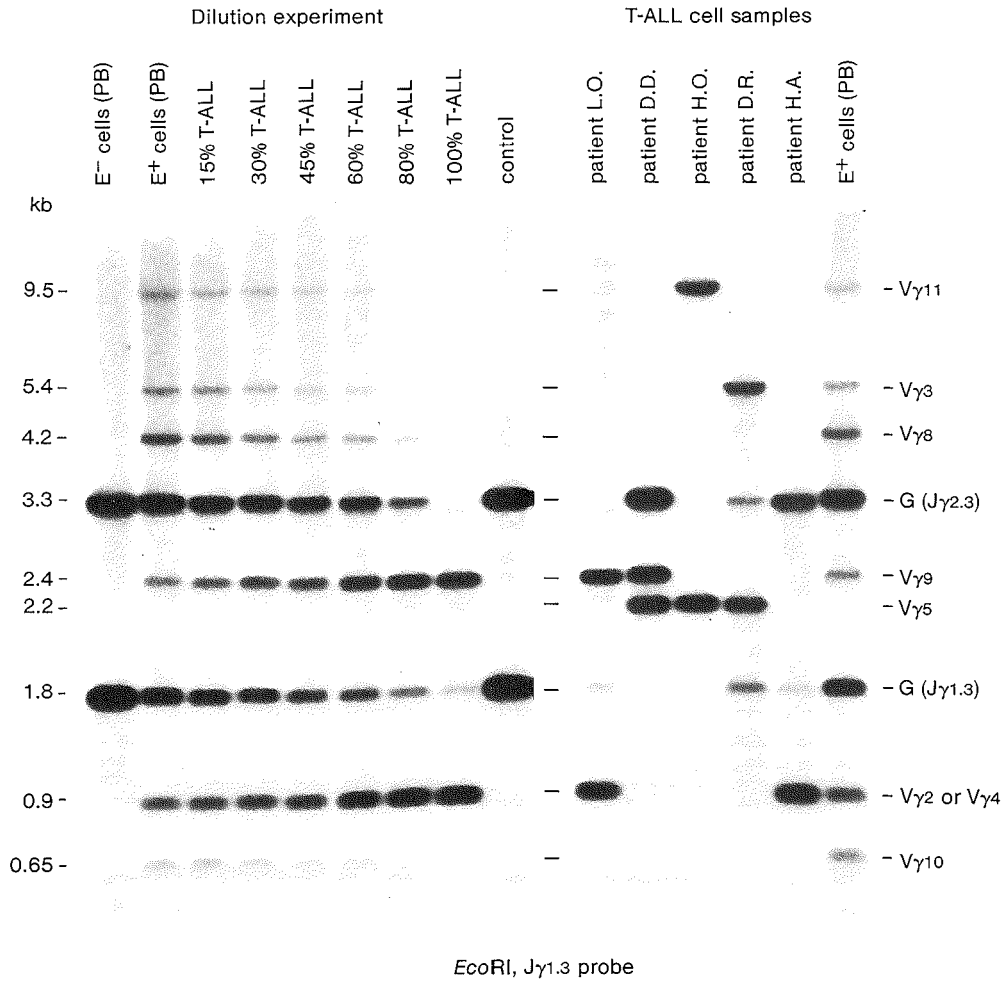


Figure 18. Analysis of the TcR-γ genes of E rosette-separated normal mononuclear blood cells (E^+ and E^- cells), T-ALL cells and a series of artificial mixtures of T-ALL cells and normal blood cells (dilution experiment). The DNA samples were digested with *Eco*RI, size-separated and blotted to a nylon membrane, which was hybridized with a ^{32}P-labeled Jγ1.3 probe. In the E^- cells (non-T cells) only the two germline bands were found, while in the E^+ cells (T cells) several rearranged bands occurred. In each T-ALL lane two rearranged bands are present. These bands have the same size as the rearranged bands in the T cell fraction of PB. In the artificial mixtures of the dilution experiment the "background" of rearranged bands of the normal T cells hampers the detection of the rearranged bands of the leukemic cells.

In most TcR-$\alpha\beta^+$ cells both alleles of the TcR-δ genes are deleted (36,98-101) and the frequency of TcR-$\gamma\delta^+$ cells in blood is relatively low (127,128). This would imply that the background of rearranged TcR-δ gene bands from normal blood T lymphocytes is low. However, it has still to be demonstrated to what extent Southern blot analysis of the TcR-δ genes is useful for the detection of a small population of malignant cells with clonally rearranged TcR-δ genes.

TABLE 6. Reported sized (in kb) of *Eco*RI and *Kpn*I restriction fragments of germline and rearranged TcR-γ genes, as detected by the Jγ1.3 probe.

Rearranged Vγ gene segments	Jγ segments				
	Jγ1.1	Jγ1.2	Jγ1.3[a]	Jγ2.1	Jγ2.3[a]
***Eco*RI digests**					
germline	ND[b]	ND	1.8	ND	3.3
Vγ2	ND	ND	0.9	ND	0.9
Vγ3	ND	ND	5.4	ND	5.4
Vγ4	ND	ND	0.9	ND	0.9
Vγ5	ND	ND	2.2	ND	2.2
ψVγ7	ND	ND	3.1	ND	3.1
Vγ8	ND	ND	4.2	ND	4.2
Vγ9	ND	ND	2.4	ND	2.4
Vγ10	ND	ND	0.65	ND	0.65
Vγ11	ND	ND	9.5	ND	9.5
***Kpn*I digests**					
germline	9.0	9.0	9.0	16.0	16.0
Vγ9	14.5	12.0	7.5	10.7	7.5
Vγ11	12.7	10.0	6.0	9.0	6.0
all other Vγ's	8.5	5.9	1.8	4.7	1.8

a. Rearrangements to Jγ1.3 and Jγ2.3 result in bands with identical size. Nevertheless it is possible to discriminate between these rearrangements, because rearrangements to Jγ2 segments will delete the TcR-γ1 locus. This deletion can easily be detected with the Cγ probe in *Eco*RI digests, but also the use of the Jγ1.3 probe in *Eco*RI digests or *Kpn*I digests will generally be informative.
b. ND, not detectable. Rearrangements to Jγ1.1, Jγ1.2 or Jγ2.1 gene segments are not detectable with the Jγ1.3 probe in *Eco*RI digests.

In diagnosis of lymphoproliferative diseases it is usually not necessary to investigate TcR-γ and TcR-δ genes. One can confine the analyses to the TcR-β genes, because they are rearranged in all TcR-$\alpha\beta^+$ T cell malignancies as well as in the majority TcR-$\gamma\delta^+$ and $CD3^-$ T cell malignancies (101,124,129,130).

Multiple rearranged bands

The analysis of Ig and TcR genes of lymphoid malignancies occasionally reveals the presence of more than two rearranged bands (131,132). This is frequently found in IgH gene analysis of acute lymphoblastic leukemias (ALL) of B cell lineage (133-137).

If more than two rearranged bands are detected, the possibility that they are due to underdigestion or polymorphisms must be excluded. Therefore, the investigation should be repeated and an additional restriction enzyme should be used, to study whether the multiple bands remain. For instance, if multiple rearranged IgH gene bands are detectable in *Eco*RI digests or *Hin*dIII digests, while only two bands are detected in *Bam*HI digests (133,134), this may be due to the presence of the HVP region at the 5´ side of the JH gene segments (Figure

5)(11). Therefore, also germline control DNA from the same patient should be used in the analyses, if available.

The presence of multiple rearranged bands may be due to chromosome aberrations (hyperdiploidy or translocations) or to the presence of subclones with differently rearranged Ig or TcR genes (131-137). Obvious differences in intensity between pairs of rearranged bands suggest oligoclonality, while the presence of three or more bands of comparable intensity may be due to hyperdiploidy. Optimal discrimination between these two causes is only possible, if also cytogenetic analysis is performed.

Detection limit of the Southern blot technique

Several studies indicate that the limit for the detection of clonal Ig or TcR gene rearrangements is about 5 to 10% (138-140), while others have reported the detection of 1% of clonal cells (8,141-143). One report even suggests that as low as 0.2% of malignant cells are detectable (144).

In most studies the detection limit was determined by mixing of DNA samples (8,140,141, 143,144). The objection against such mixing experiments is, that it is difficult to make accurate mixtures of high molecular weight DNA samples. This probably explains the discrepancies between the reported detection limits. Therefore we performed several dilution experiments, in which we did not mix DNA samples, but viable cells.

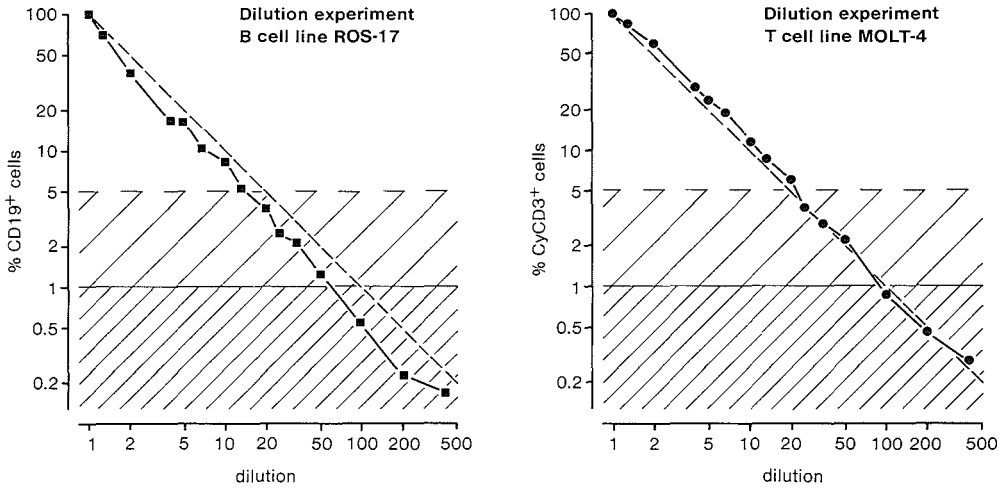


Figure 19. Dilution experiments to determine the detection limit of the Southern blot technique. The B cell line ROS-17 and the T cell line Molt-4 were mixed in various proportion. The mixtures were checked by immunologic marker analysis (CD19 as marker for ROS-17 cells and CyCD3 as marker for Molt-4 cells). DNA from each mixture was subjected to IgH gene analysis (JH probe in *Bgl*II digests) for the detection of ROS-17 cells and to TcR-β gene analysis (Jβ2 probe in *Eco*RI digests) for the detection of Molt-4 cells. These experiments showed that the detection limit of the Southern blot technique generally is 4-5%, but that it is possible to detect 1-2% of clonal cells if the exact position of the rearranged band(s) is known.

The B cell line ROS-17 and the T cell line Molt-4 were mixed in various proportions. The mixtures were checked with immunologic marker analysis: cytoplasmic CD3 (CyCD3) staining was used as marker for Molt-4 cells, while CD19 was used as marker for ROS-17 cells. DNA was extracted from each mixture and subjected to IgH gene analysis for the detection of ROS-17 cells as well as to TcR-β gene analysis for the detection of Molt-4 cells. The results of one of these experiments are summarized in Figure 19.

From the dilution experiments as well as from our experience with clinical cell samples we conclude that the detection limit of the Southern blot technique generally is 4-5%. However, if one knows the exact position of the rearranged bands, it is often possible to detect 1-2% of clonal cells (Figure 19). The latter may be important for staging of NHL at diagnosis (e.g. detection of lymphoma cells in blood and bone marrow) and for the detection of minimal residual disease during or after treatment (145-147).

DIAGNOSTIC APPLICATIONS OF Ig AND TcR GENE ANALYSIS

The various lymphoid malignancies can be regarded as the malignant counterparts of cells in the various stages of lymphoid differentiation (148-152). The ALL represent malignant counterparts of immature lymphoid cells, while the chronic lymphocytic leukemias (CLL) and the NHL represent malignant counterparts of more mature lymphoid cells (150-152). This implies that malignant lymphoid cells have virtually the same characteristics as normal lymphoid cells. Since Ig and TcR gene rearrangement occurs early during lymphoid differentiation, almost all lymphoid malignancies have rearranged Ig or TcR genes.

Generally routine morphologic techniques and immunologic marker analysis are sufficient to demonstrate the presence of a lymphoid malignancy (150-152). However, sometimes diagnostic problems arise, especially if the lymphoproliferation contains a small subpopulation of suspected B cells or if the proliferation concerns mature T lymphocytes. Although Southern blot analysis is time consuming and expensive, in selected cases analysis of Ig and TcR genes may be important to prove or exclude the clonal character of the lymphoproliferation (7,153-155).

Based on data from the literature and our own experiences during the last five years, we conclude that analysis of Ig and TcR genes can be used for several diagnostic purposes, which will be indicated below. These diagnostic applications will be discussed more extensively in Chapter 6.

Discrimination between reactive polyclonal lymphoid cell populations and a monoclonal lymphoid cell population

Using morphologic techniques or immunologic marker analysis, it may be difficult to determine whether a T cell population in a lymph node or T cell infiltrate in the skin represents normal T cells or malignant T cells. Generally, analysis of TcR-β genes allows discrimination between the presence of a polyclonal T cell population and a monoclonal T cell population (158-160).

Caution: Clonality is not always equivalent to malignancy

Clinically benign lymphoproliferation may consist of clonal cell populations, as found in benign monoclonal gammapathies (161), lymphomatoid papulosis (113) and CD8/T-γ lymphocytosis (162). Also dysregulation of the immune system, such as in patients with primary or secondary immunodeficiencies, may result in the development of monoclonal or oligoclonal B or T cell populations without evidence of malignant behavior (161,163,164). The monoclonal or oligoclonal B cell proliferation in immunodeficiency patients are generally associated with Epstein-Barr virus (EBV) (165-168). In some patients these proliferations may proceed and transform into B cell malignancies via a multistep process (169), but also regression of such proliferations has been observed in transplant patients, after withdrawal of immunosuppressive treatment (170). This indicates that clonal lymphoproliferations in immunodeficiency patients are not necessarily malignancies (161,163,164,170), although such patients have a high tendency to develop a malignancy (171).

Detection of two or more clones in a malignancy

Several studies indicate that subclones occur in 15-30% of precursor B-ALL as detected by IgH gene analysis in multiple restriction enzyme digests (132,134,137,172,173). Since glucose-6-phosphate dehydrogenase (G6PD) isotype analysis as well as cytogenetic analysis indicate that precursor B-ALL have a clonal origin (174-176), it has been concluded that subclone formation in these leukemias is due to secondary Ig gene rearrangements (135), such as D-JH replacements (177) or V to V-D-JH rearrangements (178).

Also in a part of germinal-center-cell-derived lymphomas and immunocytomas subclones are detectable upon IgH gene analysis (179-181). The IgH gene rearrangement patterns of the subclones in these lymphomas differ from the parental clone in some but not all restriction enzyme digests (180). Therefore, it is assumed that they are due to somatic mutations (180,181), which also occur in normal germinal-center cells (182).

Finally, biclonal and oligoclonal B cell malignancies have been found in immunodeficiency patients (166,167). As indicated above, these are probably derived from EBV-induced lymphoproliferations, which are transformed into B cell malignancies (169). Therefore, these patients probably suffer from multiple lymphomas, i.e. lymphomas that do not have a common clonal origin, but are caused by the same multistep transforming process.

Analysis of lymphoid malignancies at relapse and comparison with initial diagnosis

Ig and TcR gene analysis of cell samples at diagnosis and subsequent relapse(s) can prove that the reoccurrence of a lymphoid malignancy indeed represents a relapse of the same tumor. Sometimes the histomorphological characteristics of a B cell lymphoma are changed at relapse, while the IgH and IgL gene rearrangement patterns remain identical (131).

However, it is obvious that changes in IgH gene rearrangement patterns can occur at relapse in precursor B-ALL, germinal-center-cell-derived lymphomas and immunocytomas, because subclone formation frequently occurs in these malignancies (136,147,181).

Proof or exclusion of the common clonal origin of two malignant lymphoid cell populations

In some patients with a B cell malignancy a second B cell malignancy may develop or sometimes two B cell malignancies occur simultaneously in the same patient. Southern blot analysis of Ig genes can indicate whether such malignancies have a common clonal origin (131,180,181,183-185) or represent two independent malignancies (186-191). Since subclones can develop in some B cell lymphomas (179-181), extensive analysis of both IgH and IgL genes (using multiple restriction enzyme digests) are necessary to exclude a possible common clonal origin (180,181). Even lymphomas with bitypic IgL chain expression may be derived from the same clone (181), which supports the hypothesis that an oncogenic event may have taken place early during B cell differentiation (184), before IgL gene rearrangements.

Determination of the differentiation lineage of a malignancy

In some cases it is not possible, using immunologic marker analysis, to determine whether the malignant cells belong to the B cell lineage or the T cell lineage, or whether it concerns a non-lymphoid malignancy. Although Ig and TcR gene rearrangements will generally only be functional in B cells and T cells, respectively, such rearrangements have been described to occur occasionally in cells from other differentiation lineages, such as Ig gene rearrangements in T-ALL and acute myeloid leukemias (AML) and TcR gene rearrangements in precursor-B-ALL (192,193).

Due to such "lineage infidelities", the detection of Ig or TcR gene rearrangements in malignant cells does not provide definitive proof of the differentiation lineage to which the malignancy belongs. However, the presence of germline Ig and TcR gene strongly supports the non-lymphoid character of a malignancy (194,195).

Detection of low numbers of malignant cells

As discussed above, the detection limit of routinely performed Southern blot analysis is 1 to 5% (138-143). For many lymphoid malignancies, such detection limits can also be reached by use of morphologic techniques or immunologic marker analysis (196). Southern blot analysis of Ig or TcR genes may, however, be valuable for the detection of minimal residual disease in some cases (137,147).

CONCLUSION

The Southern blot technique allows a detailed analysis of the IgH and IgL genes as well as the TcR-β, TcR-γ and TcR-δ genes. However, one should be aware of the limitations and pitfalls of these analyses, especially the occurrence of genetic polymorphisms and the limited combinatorial repertoire of TcR-γ and TcR-δ genes.

In some patients morphologic techniques and immunologic marker analysis are not sufficient for diagnosing a lymphoid malignancy. In these cases, analysis of Ig and TcR genes may be of high diagnostic value, because Southern blotting is a powerful technique for determining clonality and for proving or excluding the clonal origin of two malignant lymphoid cell populations.

ACKNOWLEDGMENTS. We gratefully acknowledge Professor Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Mr. T.M. van Os (Department of Immunology, Erasmus University, Rotterdam) and Mr. C. van Dijk (Interpoint, Hapert) for excellent assistance in the preparation of the figures and Mrs. J. de Goey-van Dooren, Ms. E.P. Schenkel and Ms. H.J. de Jager for their secretarial assistance.

REFERENCES

1. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1982:545.
2. Goelz SE, Hamilton SR, Vogelstein B. Purification of DNA from formaldehyde fixed and paraffin embedded human tissue. Biochem Biophys Res Commun 1985;130:118-26.
3. Dubeau L, Chandler LA, Gralow JR, Nichols PW, Jones PA. Southern blot analysis of DNA extracted from formalin-fixed pathology specimens. Cancer Res 1986;46:2964-9.
4. Dubeau L, Weinberg K, Jones PA, Nichols PW. Studies on immunoglobulin gene rearrangement in formalin-fixed, paraffin-embedded pathology specimens. Am J Pathol 1988;130:588-94.
5. Warford A, Pringle JH, Hay J, Henderson SD, Lauder I. Southern blot analysis of DNA extracted from formol-saline fixed and paraffin wax embedded tissue. J Pathol 1988;154:313-20.
6. Fey MF, Pilkington SP, Summers C, Wainscoat JS. Molecular diagnosis of haematological disorders using DNA from stored bone marrow slides. Br J Haematol 1987;67:489-92.
7. O'Connor NTJ, Gatter KC, Wainscoat JS, Crick J, Jones DB, Delsol G, Ralfkiaer E, De Wolf-Peeters C, Angus B, Mason DY. Practical value of genotypic analysis for diagnosing lymphoproliferative disorders. J Clin Pathol 1987;40:147-50.
8. Cleary ML, Chao J, Warnke R, Sklar J. Immunoglobulin gene rearrangement as a diagnostic criterion of B-cell lymphoma. Proc Natl Acad Sci USA 1984;81:593-7.
9. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T. Somatic rearrangement of T-cell antigen receptor gene in human T-cell malignancies. Proc Natl Acad Sci USA 1985;82:1224-7.
10. Flug F, Pelicci P-G, Bonetti F, Knowles II DM, Dalla-Favera R. T-cell receptor gene rearrangements as markers of lineage and clonality in T-cell neoplasms. Proc Natl Acad Sci USA 1985;82:3460-4.
11. Trent RJ, Williams BG, Basten A. Characterisation and uses of a hypervariable DNA polymorphism associated with the human JH immunoglobulin gene locus. Immunol Cell Biol 1987;65:371-6.
12. Taub RA, Hollis GF, Hieter PA, Korsmeyer S, Waldmann TA, Leder P. Variable amplification of immunoglobulin λ light-chain genes in human populations. Nature 1983;304:172-4.
13. Pelicci PG, Subar M, Weiss A, Dalla-Favera R, Littman DR. Molecular diversity of the human T-gamma constant region genes. Science 1987;237:1051-5.
14. Bech-Hansen NT, Linsley PS, Cox DW. Restriction fragment length polymorphisms associated with immunoglobulin Cγ genes reveal linkage disequilibrium and genomic organization. Proc Natl Acad Sci USA 1983;80:6952-6.
15. Linsley PS, Bech-Hansen NT, Siminovitch L, Cox DW. Analysis of a break in chromosome 14 mapping to the region of the immunoglobulin heavy chain locus. Proc Natl Acad Sci USA 1983;80:1997-2001.
16. Lefranc M-P, Rabbitts TH. Human immunoglobulin heavy chain A2 gene allotype determination by restriction fragment length polymorphism. Nucleic Acids Res 1984;12:1303-11.
17. Bech-Hansen NT, Cox DW. Duplication of the human immunoglobulin heavy chain $gamma_2$ gene. Am J Hum Genet 1986;38:67-74.

18. Robinson MA, Kindt TJ. Segregation of polymorphic T-cell receptor genes in human families. Proc Natl Acad Sci USA 1985;82:3804-8.
19. Lefranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-600.
20. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-50.
21. Li Y, Szabo P, Posnett DN. Molecular genotypes of the human T cell receptor γ-chain. J Immunol 1988;140:1300-3.
22. Buresi C, Ghanem N, Huck S, Lefranc G, Lefranc M-P. Exon duplication and triplication in the human T-cell receptor gamma constant region genes and RFLP in French, Lebanese, Tunisian, and Black African populations. Immunogenetics 1989;29:161-72.
23. Ghanem N, Buresi C, Lefranc G, Lefranc M-P. A unique RFLP in the human T-cell receptor γ constant region gene TRGCl. Nucleic Acids Res 1989;17:471.
24. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-7.
25. Southern EM. Detection of specific sequences among DNA fragments separated by gel electrophoresis. J Mol Biol 1975;98:503-17.
26. Carle GF, Olson MV. Separation of chromosomal DNA molecules from yeast by orthogonal-field-alternation gel electrophoresis. Nucleic Acids Res 1984;12:5647-64.
27. Schwartz DC, Cantor CR. Separation of yeast chromosome-sized DNAs by pulsed field gradient gel electrophoresis. Cell 1984;37:67-75.
28. Carle GF, Frank M, Olson MV. Electrophoretic separations of large DNA molecules by periodic inversion of the electric field. Science 1986;232:65-8.
29. Lai E. Birren BW, Clark SM, Simon ML, Hood L. Pulsed field gel electrophoresis. Bio Techniques 1989;7:34-42.
30. Grunstein M, Thomas P, Wahl GM. Transfer and immobilization of nucleic acids to S&S solid supports. Dassel: Schleicher and Schuell, 1987:34.
31. Cohen SN, Chang ACY, Hsu L. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherisia coli* by R-factor DNA. Proc Natl Acad Sci USA 1972;69:2110-4.
32. Hanahan D. Techniques for transformation of *E. coli.* In: Glover DM, ed. DNA cloning, Volume I: a practical approach. Oxford: IRL Press:109-35.
33. Maniatis T, Jeffrey A, Kleid DG. Nucleotide sequence of the rightward operator of phage λ. Proc Natl Acad Sci USA 1975;72:1184-8.
34. Rigby PWJ, Dieckmann M, Rhodes C, Berg P. Labeling deoxyribonucleic acid to high specific activity *in vitro* by nick translation with DNA polymerase I. J Mol Biol 1977;113:237-51.
35. Feinberg AP, Vogelstein B. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytic Biochem 1983;132:6-13. Addendum: Analytic Biochem 1984;137:266-7.
36. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-8.
37. Champagne E, Sagman U, Biondi A, Lewis WH, Mak TW, Minden MD. Structure and rearrangement of the T cell receptor J alpha locus in T cells and leukemic T cell lines. Eur J Immunol 1988;18:1033-8.
38. Hayday AC, Diamond DJ, Tanigawa G, Heilig JS, Folsom V, Saito H, Tonegawa S. Unusual organization and diversity of T-cell receptor α-chain genes. Nature 1985;316:828-32.
39. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.
40. Griesser H, Champagne E, Tkachuk D, Takihara Y, Lalande M, Baillie E, Minden M, Mak TW. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. Eur J Immunol 1988;18:641-4.
41. Takahashi N, Nakai S, Honjo T. Cloning of human immunoglobulin μ gene and comparison with mouse μ gene. Nucleic Acids Res 1980;8:5983-91.
42. Ravetch JV, Siebenlist U, Korsmeyer S, Waldmann T, Leder P. Structure of the human immunoglobulin μ

locus: characterization of embryonic and rearranged J and D genes. Cell 1981;27:583-91.
43. Rabbitts TH, Forster A, Milstein CP. Human immunoglobulin heavy chain genes: evolutionary comparisons of Cμ, Cδ and Cγ genes and associated switch sequences. Nucleic Acids Res 1981;9:4509-24.
44. Foroni L, Catovsky D, Rabbitts TH, Luzzatto L. DNA rearrangements of immunoglobulin genes correlate with phenotypic markers in B-cell malignancies. Mol Biol Med 1984;2:63-79.
45. Brown LA, Liu C, Berenson JR, Garcia CR, Wang R, Calame KL. Immunoglobulin JH, Cμ, and Cγ gene rearrangements in human B lymphocytes clonally transformed by Epstein-Barr virus. Proc Natl Acad Sci USA 1985;82:556-60.
46. Webb CF, Cooper MD, Burrows PD, Griffin JA. Immunoglobulin gene rearrangements and deletions in human Epstein-Barr virus-transformed cell lines producing different IgG and IgA subclasses. Proc Natl Acad Sci USA 1985;82:5495-9.
47. Borzillo GV, Cooper MD, Kubagawa H, Landay A, Burrows PD. Isotype switching in human B lymphocyte malignancies occurs by DNA deletion: evidence for nonspecific switch recombination. J Immunol 1987;139:1326-35.
48. Siminovitch KA, Jensen JP, Epstein AL, Korsmeyer SJ. Immunoglobulin gene rearrangements and expression in diffuse histiocytic lymphomas reveal cellular lineage, molecular defects, and sites of chromosomal translocation. Blood 1986;67:391-7.
49. Huck S, Keyeux G, Ghanem N, Lefranc M-P, Lefranc G. A gamma 3 hinge region probe: first specific human immunoglobulin subclass probe. FEBS Letters 1986;208:221-30.
50. Flanagan JG, Rabbitts TH. Arrangement of human immunoglobulin heavy chain constant region genes implies evolutionary duplication of a segment containing γ, ϵ and α genes. Nature 1982;300:709-13.
51. Ellison JW, Berson BJ, Hood LE. The nucleotide sequence of a human immunoglobulin Cγ1 gene. Nucleic Acids Res 1982;10:4071-9.
52. Ellison J, Hood L. Linkage and sequence homology of two human immunoglobulin γ heavy chain constant region genes. Proc Natl Acad Sci USA 1982;79:1984-8.
53. Krawinkel U, Rabbitts TH. Comparison of the hinge-coding segments in human immunoglobulin gamma heavy chain genes and the linkage of the gamma 2 and gamma 4 subclass genes. EMBO J 1982;1:403-7.
54. Takahashi N, Ueda S, Obata M, Nikaido T, Nakai S, Honjo T. Structure of human immunoglobulin gamma genes: implications for evolution of a gene family. Cell 1982;29:671-9.
55. Huck S, Fort P, Crawford DH, Lefranc M-P, Lefranc G. Sequence of a human immunoglobulin gamma 3 heavy chain constant region gene: comparison with the human Cγ genes. Nucleic Acids Res 1986;14:1779-89.
56. Ravetch JV, Kirsch IR, Leder P. Evolutionary approach to the question of immunoglobulin heavy chain switching: evidence from cloned human and mouse genes. Proc Natl Acad Sci USA 1980;77:6734-8.
57. Flanagan JG, Lefranc M-P, Rabbitts TH. Mechanisms of divergence and convergence of the human immunoglobulin α1 and α2 constant region gene sequences. Cell 1984;36:681-8.
58. Flanagan JG, Rabbitts TH. The sequence of a human immunoglobulin episilon heavy chain constant region gene, and evidence for three non-allelic genes. EMBO J 1982;1:655-60.
59. Nishida Y, Miki T, Hisajima H, Honjo T. Cloning of human immunoglobulin ϵ chain genes: evidence for multiple Cϵ genes. Proc Natl Acad Sci USA 1982;79:3833-7.
60. Max EE, Battey J, Ney R, Krisch I, Leder P. Duplication and deletion in the human immunoglobulin ϵ genes. Cell 1982;29:691-9.
61. Battey J, Max EE, McBride WO, Swan D, Leder P. A processed human immunoglobulin ϵ gene has moved to chromosome 9. Proc Natl Acad Sci USA 1982;79:5956-60.
62. Hisajima H, Nishida Y, Nakai S, Takahashi N, Ueda S, Honjo T. Structure of the human immunoglobulin Cϵ2 gene, a truncated pseudogene: implications for its evolutionary origin. Proc Natl Acad Sci USA 1983;80:2995-9.
63. White MG, Shen AL, Word CJ, Tucker PW, Blattner FR. Human immunoglobulin D: genomic sequence of the delta heavy chain. Science 1985;228:733-7.
64. Lefranc M-P, Lefranc G, Rabbitts TH. Inherited deletion of immunoglobulin heavy chain constant region genes in normal human individuals. Nature 1982;300:760-2.
65. Lefranc M-P, Lefranc G, De Lange G, Out TA, Van den Broek PJ, Van Nieuwkoop J, Radl J, Helal AN,

Chaabani H, Van Loghem E, Rabbitts TH. Instability of the human immunoglobulin heavy chain constant region locus indicated by different inherited chromosomal deletions. Mol Biol Med 1983;1:207-17.
66. Smith CIE, Hammerström L, Henter J-I, De Lange GG. Molecular and serologic analysis of IgG1 deficiency caused by new forms of the constant region of the Ig H chain gene deletions. J Immunol 1989;142:4514-9.
67. Migone N, Oliviero S, De Lange G, Delacroix DL, Boschis D, Altruda F, Silengo L, DeMarchi M, Carbonara AO. Multiple gene deletions within the human immunoglobulin heavy-chain cluster. Proc Natl Acad Sci USA 1984;81:5811-5.
68. Chaabani H, Bech-Hansen NT, Cox DW. A multigene deletion within the immunoglobulin heavy-chain region. Am J Hum Genet 1985;37:1164-71.
69. Lefranc M-P, Lefranc G. Human immunoglobulin heavy-chain multigene deletions in healthy individuals. FEBS Letters 1987;213:231-7.
70. Bottaro A, De Marchi M, De Lange G, Boccazzi C, Caldesi F, Gallina R, Carbonara AO. New types of multiple and single gene deletions in the human IgCH locus. Immunogenetics 1989;29:44-8.
71. Bottaro A, De Marchi M, De Lange GG, Carbonara AO. Gene deletions within the human immunoglobulin heavy chain constant region gene cluster. Exp Clin Immunogenet 1989;6:55-9.
72. Hieter PA, Max EE, Seidman JG, Maizel JV, Leder P. Cloned human and mouse kappa immunoglobulin constant and J region genes conserve homology in functional segments. Cell 1980;22:197-207.
73. Hieter PA, Maizel JV, Leder P. Evolution of human immunoglobulin κ J region genes. J Biol Chem 1982;257:1516-22.
74. Hieter PA, Korsmeyer SJ, Waldmann TA, Leder P. Human immunoglobulin κ light-chain genes are deleted or rearranged in λ-producing B cells. Nature 1981;290:368-72.
75. Korsmeyer SJ, Hieter PA, Sharrow SO, Goldman CK, Leder P, Waldmann TA. Normal human B cells display ordered light chain gene rearrangements and deletions. J Exp Med 1982;156:975-85.
76. Siminovitch KA, Bakhshi A, Goldman P, Korsmeyer SJ. A uniform deleting element mediates the loss of κ genes in human B cells. Nature 1985;316:260-2.
77. Graninger WB, Goldman PL, Morton CC, O'Brien SJ, Korsmeyer SJ. The κ-deleting element: germline and rearranged, duplicated and dispersed forms. J Exp Med 1988;167:488-501.
78. Klobeck H-G, Zachau HG. The human Cκ gene segment and the kappa deleting element are closely linked. Nucleic Acids Res 1986;14:4591-603.
79. Hieter PA, Hollis GF, Korsmeyer SJ, Waldmann TA, Leder P. Clustered arrangement of immunoglobulin λ constant region genes in man. Nature 1981;294:536-40.
80. Udey JA, Blomberg B. Human λ light chain locus: organization and DNA sequences of three genomic J regions. Immunogenetics 1987;25:63-70.
81. Dariavach P, Lefranc G, Lefranc M-P. Human immunoglobulin Cλ6 gene encodes the $Kern^{+}Oz^{-}$ λ chain and Cλ4 and Cλ5 are pseudogenes. Proc Natl Acad Sci USA 1987;84:9074-8.
82. Hollis GF, Hieter PA, McBride OW, Swan D, Leder P. Processed genes: a dispersed human immunoglobulin gene bearing evidence of RNA-type processing. Nature 1982;296:321-5.
83. Chang H, Dmitrovsky E, Hieter PA, Mitchell K, Leder P, Turoczi L, Kirsch IR, Hollis GF. Identification of three new Ig λ-like genes in man. J Exp Med 1986;163:425-35.
84. Hollis GF, Evans RJ, Stafford-Hollis JM, Korsmeyer SJ, McKearn JP. Immunoglobulin λ light-chain-related genes 14.1 and 16.1 are expressed in pre-B cells and may encode the human immunoglobulin ω light-chain protein. Proc Natl Acad Sci USA 1989;86:5552-6.
85. Sims JE, Tunnacliffe A, Smith WJ, Rabbitts TH. Complexity of human T-cell antigen receptor β-chain constant- and variable-region genes. Nature 1984;312:541-5.
86. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-6.
87. Tunnacliffe A, Rabbitts TH. Sequence of the Dβ2-Jβ2 region of the human T-cell receptor β-chain locus. Nucleic Acids Res 1985;13:6651-61.
88. Toyonaga B, Yorshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8.
89. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.

90. Tunnacliffe A, Kefford R, Milstein C, Forster A, Rabbitts TH. Sequence and evolution of the human T-cell antigen receptor β-chain genes. Proc Natl Acad Sci USA 1985;82:5068-72.
91. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
92. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
93. Huck S, Lefranc M-P. Rearrangements to the JP1, JP and JP2 segments in the human T-cell rearranging gamma gene (TRGγ) locus. FEBS Letters 1987;224:291-6.
94. Lefranc M-P, Rabbitts TH. Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types. Nature 1985;316:464-6.
95. Quertermous T, Murre C, Dialynas D, Duby AD, Strominger JL, Waldman TA, Seidman JG. Human T-cell γ chain genes: organization, diversity, and rearrangement. Science 1986;231:252-5.
96. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germline variable genes belonging to two subgroups. Cell 1986;45:237-46.
97. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-8.
98. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-8.
99. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-82.
100. Foroni L, Laffan M, Boehm T, Rabbitts TH, Catovsky D, Luzzatto L. Rearrangement of the T-cell receptor δ genes in human T-cell leukemias. Blood 1989;73:559-65.
101. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334:42.
102. Okazaki K, Sakano H. Thymocyte circular DNA excised from T cell receptor α-δ gene complex. EMBO J 1988;7:1669-74.
103. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-7.
104. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-94.
105. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-101.
106. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-70.
107. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. The human T-cell receptor delta chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
108. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-4.
109. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ. T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
110. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 1985;313:776-83.
111. Berliner N, Duby AD, Linch DC, Murre C, Quertermous T, Knott LJ, Azin T, Newland AC, Lewis DL, Galvin MC, Seidman JG. T cell receptor gene rearrangements define a monoclonal T cell proliferation in patients with T cell lymphocytosis and cytopenia. Blood 1986;67:914-8.
112. Pittaluga S, Raffeld M, Lipford EH, Cossman J. 3A1 (CD7) expression precedes Tβ gene rearrangements in

precursor T (lymphoblastic) neoplasms. Blood 1986;68:134-9.
113. Weiss LM, Wood GS, Trela M, Warnke RA, Sklar J. Clonal T-cell populations in lymphomatoid papulosis: evidence of a lymphoproliferative origin for a clinically benign disease. N Engl J Med 1986;315:475-9.
114. Kadin ME, Vonderheid EC, Sako D, Clayton LK, Olbricht S. Clonal composition of T cells in lymphomatoid papulosis. Am J Pathol 1987;126:13-7.
115. LeBoit PE, Parslow TG. Gene rearrangements in lymphoma: applications to dermatopathology. Am J Dermatopathol 1987;9:212-8.
116. Uppenkamp M, Pittaluga S, Lipford EH, Cossman J. Limited diversity and selection of rearranged γ genes in polyclonal T cells. J Immunol 1987;138:1618-20.
117. Lefranc, M-P. The human T-cell rearranging gamma (TRG) genes and the gamma T-cell receptor. Biochimie 1988;70:901-8.
118. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc MP, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-83.
119. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
120. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
121. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-8.
122. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.
123. Boehm TLJ, Werle A, Ganser A, Kornhuber B, Drahovsky D. T cell receptor γ chain variable gene rearrangements in acute lymphoblastic leukemias of T and B lineage. Eur J Immunol 1987;17:1593-7.
124. Greenberg JM, Quertermous T, Seidman JG, Kersey JH. Human T cell γ-chain gene rearrangements in acute lymphoid and nonlymphoid leukemia: comparison with the T cell receptor β-chain gene. J Immunol 1986;137:2043-9.
125. Le Paslier D, Chen Z, Loiseau P, Cohen D, Sigaux F. T cell rearranging gene γ: diversity and mRNA expression in fresh cells from T cell acute lymphoblastic leukemia. Blood 1987;70:637-46.
126. Subar M, Pelicci PG, Neri A, Allavena P, Littman DR, Knowles II DM, Dalla-Favera R. Patterns of T cell receptor gamma gene rearrangement and expression in B and T lymphoid malignancies. Leukemia 1988;2:19-26.
127. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
128. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.
129. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
130. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Comparison of T cell receptor α, β, and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J Clin Invest 1988;81:989-96.
131. Siegelman MH, Cleary ML, Warnke R, Sklar J. Frequent biclonality and Ig gene alterations among B cell lymphomas that show multiple histologic forms. J Exp Med 1985;161:850-63.
132. Kitchingman GR, Mirro J, Stass S, Rovigatti U, Melvin SL, Williams DL, Raimondi SC, Murphy SB. Biologic and prognostic significance of the presence of more than two μ heavy-chain genes in childhood acute lymphoblastic leukemia of B precursor cell origin. Blood 1986;67:698-703.
133. Mirro J, Kitchingman G, Williams D, Lauzon GJ, Lin C-C, Callihan T, Zipf TF. Clinical and laboratory characteristics of acute leukemia with the 4;11 translocation. Blood 1986;67:689-97.
134. Rechavi G, Brok-Simoni F, Katzir N, Mandel M, Umiel T, Stark B, Zaizov R, Ben-Bassat I, Ramot B. More than

two immunoglobulin heavy chain J region genes in the majority of infant leukemia. Leukemia 1988;2:347-50.
135. Bird J, Galili N, Link M, Stites D, Sklar J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukemia. J Exp Med 1988;168:229-45.
136. Raghavachar A, Thiel E, Bartram CR. Analyses of phenotype and genotype in acute lymphoblastic leukemias at first presentation and in relapse. Blood 1987;70:1079-83.
137. Katz F, Ball L, Gibbons B, Chessells J. The use of DNA probes to monitor minimal residual disease in childhood acute lymphoblastic leukemia. Br J Haematol 1989;73:173-80.
138. Arnold A, Cossman J, Bakhshi A, Jaffe ES, Waldmann TA, Korsmeyer SJ. Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J Med 1983;309:1593-9.
139. O'Connor NTJ. Genotypic analysis of lymph node biopsies. J Pathol 1987;151:185-90.
140. Aisenberg AC, Wilkes BM, Jacobson JO, Harris NL. Immunoglobulin gene rearrangements in adult non-Hodgkin's lymphoma. Am J Med 1987;82:738-44.
141. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T. Somatic rearrangement of T-cell antigen receptor gene in human T-cell malignancies. Proc Natl Acad Sci USA 1985;82:1224-7.
142. Berliner N, Ault KA, Martin P, Weinberg DS. Detection of clonal excess in lymphoproliferative disease by κ/λ analysis: correlation with immunoglobulin gene DNA rearrangement. Blood 1986;67:80-5.
143. Sundeen J, Lipford E, Uppenkamp M, Sussman E, Wahl L, Raffeld M, Cossman J. Rearranged antigen receptor genes in Hodgkin's disease. Blood 1987;70:96-103 and Errata: Blood 1987;70:893-4.
144. Zehnbauer BA, Pardoll DM, Burke PJ, Graham ML, Vogelstein B. Immunoglobulin gene rearrangements in remission bone marrow specimens from patients with acute lymphoblastic leukemia. Blood 1986;67:835-8.
145. Hu E, Thompson J, Horning S, Trela M, Lowder J, Levy R, Sklar J. Detection of B-cell lymphoma in peripheral blood by DNA hybridisation. Lancet 1985;ii:1092-5.
146. Hu E, Horning S, Flynn S, Brown S, Warnke R, Sklar J. Diagnosis of B cell lymphoma by analysis of immunoglobulin gene rearrangements in biopsy specimens obtained by fine needle aspiration. J Clin Oncol 1986;4:278-83.
147. Wright JJ, Poplack DG, Bakhshi A, Reaman G, Cole D, Jensen JP, Korsmeyer SJ. Gene rearrangements as markers of clonal variation and minimal residual disease in acute lymphoblastic leukemia. J Clin Oncol 1987;5:735-41.
148. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
149. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
150. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
151. Greaves MF. Differentiation-linked leukemogenesis in lymphocytes. Science 1986;234:697-704.
152. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.
153. Korsmeyer SJ. Antigen receptor genes as molecular markers of lymphoid neoplasms. J Clin Invest 1987;79:1291-5.
154. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.
155. Sklar J, Weiss LM. Applications of antigen receptor gene rearrangement to the diagnosis and characterization of lymphoid neoplasms. Ann Rev Med 1988;39:315-34.
156. Aisenberg AC, Krontiris TG, Mak TW, Wilkes BM. Rearrangement of the gene for the beta chain of the T-cell receptor in T-cell chronic lymphocytic leukemia and related disorders. N Engl J Med 1985;313:529-33.
157. Weiss LM, Hu E, Wood GS, Moulds C, Cleary ML, Warnke R, Sklar J. Clonal rearrangements of T-cell receptor genes in mycosis fungoides and dermatopathic lymphadenopathy. N Engl J Med 1985;313:539-44.
158. Cleary ML, Trela MJ, Weiss LM, Warnke R, Sklar J. Most null large cell lymphomas are B lineage neoplasms. Lab Invest 1985;53:521-5.
159. De Jong D, Voetdijk BMH, Van Ommen GJB, Kluin-Nelemans JC, Beverstock GC, Kluin PM. Translocation t(14;18) in B cell lymphomas as a cause for defective immunoglobulin production. J Exp Med 1989;169:613-

24.
160. Ramsay AD, Smith WJ, Isaacson PG. T-cell-rich B-cell lymphoma. Am J Surg Path 1988;12:433-43.
161. Gerritsen E, Vossen J, Van Tol M, Jol-Van der Zijde C, Van der Weijden-Ragas R, Radl J. Monoclonal gammopathies in children. J Clin Immunol 1989;9:296-305.
162. Oshimi K. Granular lymphocyte proliferative disorders: report of 12 cases and review of the literature. Leukemia 1988;2:617-27.
163. Frenkel J, Neijens HJ, Den Hollander JC, Wolvers-Tettero ILM, Van Dongen JJM. Oligoclonal T cell proliferative disorder in combined immunodeficiency. Pediatr Res 1988;24:622-7.
164. Radl J, Valentijn RM, Haaijman JJ, Paul LC. Monoclonal gammapathies in patients undergoing immunosuppressive treatment after renal transplantation. Clin Immunol Immunopathol 1985;37:98-102.
165. Cleary ML, Sklar J. Lymphoproliferative disorders in cardiac transplant recipients are multiclonal lymphomas. Lancet 1984;ii:489-93.
166. Shearer WT, Ritz J, Finegold MJ, Guerra IC, Rosenblatt HM, Lewis DE, Pollack MS, Taber LH, Sumaya CV, Grumet FC, Cleary ML, Warnke R, Sklar J. Epstein-Barr virus-associated B-cell proliferations of diverse clonal origins after bone marrow transplantation in a 12-year-old patient with severe combined immunodeficiency. N Eng J Med 1985;312:1151-9.
167. Pelicci P-G, Knowles II DM, Arlin ZA, Wieczorek R, Luciw P, Dina D, Basilico C, Dalla-Favera R. Multiple monoclonal B cell expansions and *c-myc* oncogene rearrangements in acquired immune deficiency syndrome-related lymphoproliferative disorders. J Exp Med 1986;164:2049-76.
168. Knowles DM, Chamulak GA, Subar M, Burke JS, Dugan M, Wernz J, Slywotzky C, Pelicci P-G, Dalla-Favera R, Raphael B. Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS): the New York university medical center experience with 105 patients (1981-1986). Ann Intern Med 1988;108:744-53.
169. Hanto DW, Frizzera G, Gajl-Peczalska KJ, Simmons RL. Epstein-Barr virus, immunodeficiency, and B cell lymphoproliferation. Transplantation 1985;39:461-72.
170. Starzl TE, Porter KA, Iwatsuki S, Rosenthal JT, Shaw BW, Atchison RW, Nalesnik MA, Ho M, Griffith BP, Hakala TR, Hardesty RL, Jaffe R, Bahnson HT. Reversibility of lymphomas and lymphoproliferative lesions developing under cyclosporin-steroid therapy. Lancet 1984;i:583-7.
171. Spector BD, Perry III GS, Kersey JH. Genetically determined immunodeficiency diseases (GDID) and malignancy: report from the immunodeficiency-cancer registry. Clin Immunol Immunopathol 1978;11:12-29.
172. Foa R, Migone M, Saitta M, Fierro MT, Giubellino MC, Lusso P, Cordero di Montezemolo L, Miniero R, Lauria F. Different stages of B cell differentiation in non-T acute lymphoblastic leukemia. J Clin Invest 1984;74:1756-63.
173. Raghavachar A, Bartram CR, Ganser A, Heil G, Kleihauer E, Kubanek B. Acute undifferentiated leukemia: implications for cellular origin and clonality suggested by analysis of surface markers and immunoglobulin gene rearrangement. Blood 1986;68:658-62.
174. Dow LW, Martin P, Moohr J, Greenberg M, Macdougall LG, Najfeld V, Fialkow PJ. Evidence for clonal development of childhood acute lymphoblastic leukemia. Blood 1985;66:902-7.
175. Williams DL, Look T, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S. New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia. Cell 1984;36:101-9.
176. Uckun FM, Gajl-Peczalska KJ, Provisor AJ, Heerema NA. Immunophenotype-karyotype associations in human acute lymphoblastic leukemia. Blood 1989;73:271-80.
177. Reth MG, Jackson S, Alt FW. VHDJH formation and DJH replacement during pre-B differentiation: non-random usage of gene segments. EMBO J 1986;5:2131-8.
178. Reth M, Gehrmann P, Petrac E, Wiese P. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. Nature 1986;322:840-2.
179. Raffeld M, Wright JJ, Lipford E, Cossman J, Longo DL, Bakhshi A, Korsmeyer SJ. Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. Cancer Res 1987;47:2537-42.
180. Cleary ML, Galili N, Trela M, Levy R, Sklar J. Single cell origin of bigenotypic and biphenotypic B cell proliferations in human follicular lymphomas. J Exp Med 1988;167:582-97.
181. De Jong D, Voetdijk BMH, Van Ommen GJB, Kluin PM. Alterations in immunoglobulin genes reveal the origin

and evolution of monotypic and bitypic B cell lymphomas. Am J Pathol 1989;134:1233-42.
182. Slekevitz M, Kocks C, Rajewsky K, Dildrop R. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 1987;48:757-70.
183. Bertoli LF, Kubagawa H, Borzillo GV, Mayumi M, Prchal JT, Kearney JF, Durant JR, Cooper MD. Analysis with antiidiotype antibody of a patient with chronic lymphocytic leukemia and a large cell lymphoma (Richter's syndrome). Blood 1987;70:45-50.
184. De Jong D, Voetdijk BMH, Beverstock GC, Van Ommen GJB, Willemze R, Kluin PM. Activation of the c-*myc* oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. N Engl J Med 1988;318:1373-8.
185. Michiels JJ, Van Dongen JJM, Hagemeijer A, Sonneveld P, Ploemacher RE, Adriaansen HJ, Van der Kwast ThH, Brederoo P, Abels J. Richter's syndrome with identical immunoglobulin gene rearrangements in the chronic lymphocytic leukemia and the supervening non-Hodgkin lymphoma. Leukemia 1989;3:819-24.
186. Van Dongen JJM, Hooijkaas H, Michiels JJ, Grosveld G, De Klein A, Van der Kwast ThH, Prins MEF, Abels J, Hagemeijer A. Richter's syndrome with different immunoglobulin light chains and different heavy chain gene rearrangements. Blood 1984;64:571-5.
187. Giardina SL, Schroff RW, Woodhouse CS, Golde DW, Oldham RK, Cleary ML, Sklar J, Pritikin N, Foon KA. Detection of two distinct malignant B cell clones in a single patient using anti-idiotype monoclonal antibodies and immunoglobulin gene rearrangement. Blood 1985;66:1017-21.
188. Downing JR, Grossi CE, Smedberg CT, Burrows PD. Diffuse large cell lymphoma in a patient with hairy cell leukemia: immunoglobulin gene analysis reveals separate clonal origins. Blood 1986;67:739-44.
189. McDonnell JM, Beschorner WE, Staal SP, Spivak JL, Mann RB. Richter's syndrome with two different B-cell clones. Cancer 1986;58:2031-7.
190. Ben-Ezra J, Winberg CD, Wu A, Sheibani K, Rappaport H. Concurrent presence of two clonal populations in small lymphocytic lymphoma of the lung. Hum Pathol 1987;18:399-402.
191. Ostrowski M, Minden M, Wang C, Bailsy D. Immunophenotypic and gene probe analysis of a case of Richter's syndrome. Am J Clin Pathol 1989;91:215-21.
192. Greaves MF, Furley AJW, Chan LC, Ford AM, Molgaard HV. Inappropriate rearrangement of immunoglobulin and T-cell receptor genes. Immunol Today 1987;8:115-6.
193. Furley AJW, Chan LC, Mizutani S, Ford AM, Weilbaecher K, Pegram SM, Greaves MF. Lineage specificity of rearrangement and expression of genes encoding the T cell receptor-T3 complex and immunoglobulin heavy chain in leukemia. Leukemia 1987;1:644-52.
194. Meyers FJ, Miller CH, Lewis JP. The resolution of phenotypic ambiguity in a case of human leukemia using cytogenetics and immunoglobulin gene rearrangement. Am J Clin Pathol 1986;85:372-4.
195. Lubinski J, Chosia M, Huebner K. Molecular genetic analysis in the diagnosis of lymphoma in fine needle aspiration biopsies. II. Lymphomas versus nonlymphoid malignant tumors. Anal Quant Cytol Histol 1988;10:399-404.
196. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hahlen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Lowenberg B, eds. Minimal Residue disease in acute leukemia. Dordrecht: M. Nijhoff, 1986: 113-33.

CHAPTER 4

HUMAN T CELL DIFFERENTIATION

CHAPTER 4.1

INTRODUCTION: DEVELOPMENT OF HUMAN T LYMPHOCYTES AND THEIR THYMUS-DEPENDENCY*

Jacques J.M. van Dongen[1], W. Marieke Comans-Bitter[1,2], Ingrid L.M. Wolvers-Tettero[1] and Jannie Borst[3]

1. Department of Immunology, Erasmus University/ University Hospital Dijkzigt, Rotterdam, The Netherlands;
2. Department of Pediatrics, Sophia Children's Hospital, Rotterdam, The Netherlands;
3. Division of Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands.

INTRODUCTION

It is generally assumed that the earliest precursors of T lymphocytes are generated in the bone marrow (1-4). Thymus repopulation experiments in mice have demonstrated that these precursor T cells, the prothymocytes, are able to migrate to the thymus where they can develop into mature T lymphocytes (1-4).

Most data on human T cell differentiation are derived from studies on normal bone marrow (BM), thymus and blood samples as well as from analysis of T cell acute lymphoblastic leukemias (T-ALL). T-ALL can be regarded as malignant clonal expansions of cells at the various stages of T cell differentiation and thus represent a convenient tool for studying the phenotypic and genotypic changes during T cell development (5-7).

One of the most important events during T cell development is the rearrangement of the genes encoding the T cell receptor (TcR)(8,9). The TcR consists of two chains, closely associated with the CD3 protein complex (TcR-CD3)(8-10). Two main types of TcR have been recognized: the "classical" TcR-$\alpha\beta$ (8,9) and the "alternative" TcR-$\gamma\delta$ (11, 12). The majority of mature T lymphocytes in blood and lymphoid tissues express TcR-$\alpha\beta$, a minority TcR-$\gamma\delta$ (13,14).

Another important process during T cell differentiation is the elimination of T cells recognizing self antigens (negative selection) and the positive selection of T cells for recognition of antigens in de context of self major histocompatibility complex (MHC) molecules (9). This selection occurs in the thymus and at least concerns TcR-$\alpha\beta^+$ cells (9,15). The T cell accessory molecules CD4 and CD8 play a supportive role in this MHC-restricted antigen recognition (16,17). TcR-$\gamma\delta^+$ T lymphocytes probably also recognize antigen in association with MHC or MHC-like molecules (18-20), but it is unclear to what extent intrathymic differentiation and thymic selection of TcR-$\gamma\delta^+$ cells occurs (21).

In this review we shall summarize our data concerning the immunophenotype of normal cells and leukemic cells (7,14,22-28), TcR gene rearrangements in T-ALL (26,29-32), TcR-$\alpha\beta$

* *Published in: Thymus 1990, in press.*

and TcR-$\gamma\delta$ expression in healthy children and children with hypoplasia or aplasia of the thymus (DiGeorge anomaly)(33), and the repertoire of normal TcR-$\gamma\delta^+$ T lymphocytes (28,33). Based on these data and those from the literature, we shall discuss the immunophenotype of T cells during T cell differentiation, the hierarchic order of TcR gene rearrangement and the thymus-dependency of TcR-$\gamma\delta^+$ cell development.

IMMUNOPHENOTYPE OF T CELLS DURING DIFFERENTIATION

Based on immunologic marker analysis of T-ALL cell samples a hypothetical scheme of T cell differentiation has been designed, as illustrated in Figure 1 (22,26,27,29,34). The enzyme terminal deoxynucleotidyl transferase (TdT) is present in the nucleus of immature lymphoid cells (35). Virtually all T-ALL and their assumed normal counterparts, i.e. cortical thymocytes, express TdT, while mature T lymphocytes are negative for this enzyme (5-7). This expression pattern is related to the presumed function of TdT to insert nucleotides at the joining sites of gene segments during TcR gene rearrangements (36-38).

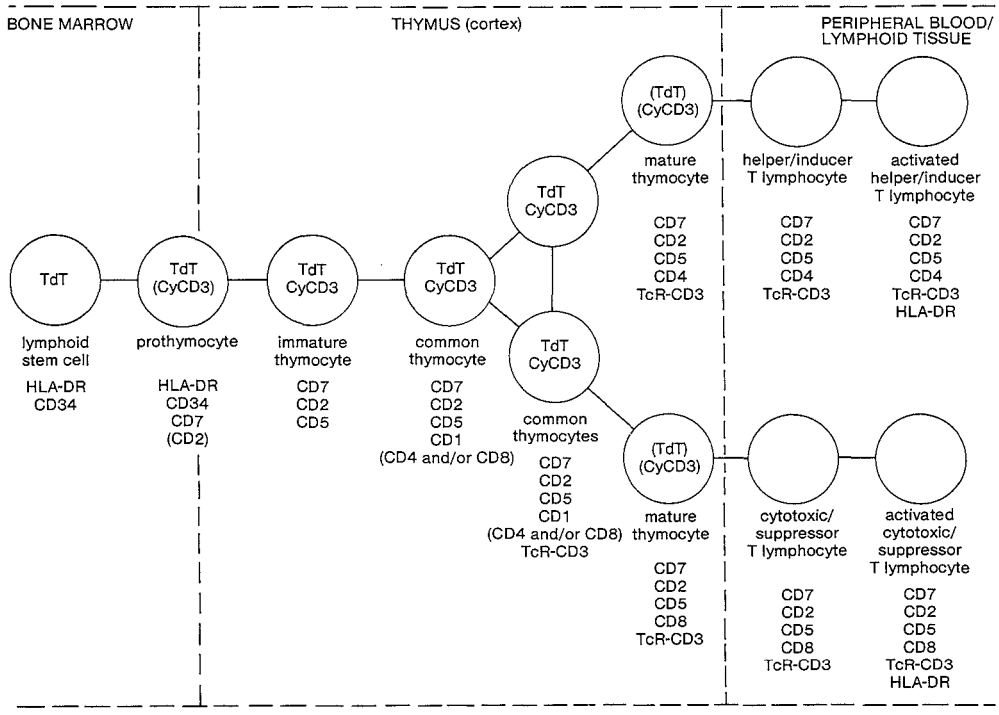


Figure 1. Hypothetical scheme of human T cell differentiation based on immunophenotypic analysis of T-ALL and infant thymocytes. The immunophenotype of cells in the various stages of T cell differentiation are indicated (22,26,27,34). Immunologic markers in parentheses are not always expressed. The dotted lines represent the tissue compartments where the various cell types are found.

The majority of lymphoid precursor cells in bone marrow express both HLA-DR and the precursor antigen CD34, markers also expressed by "prothymocytic" T-ALL. This type of T-ALL was first recognized as an ALL, which expressed HLA-DR and the pan-T cell antigens CD7 and CD2, but appeared to be negative for all other B and T cell antigens tested (22,24). Double and triple immunofluorescence (IF) stainings revealed that normal counterparts of this immature T-ALL occur in low frequencies in BM and thymus (23,24).

The pan-T cell markers CD7 and CD2 are expressed by virtually all T cells throughout development, the CD5 antigen is present on most cortical thymocytes and mature T lymphocytes, while the expression of the CD1 antigen is restricted to the "common thymocytes" in the thymic cortex (34). A large part of cortical thymocytes express CD4 and/or CD8, while the majority of peripheral T lymphocytes express CD4 or CD8 in a mutually exclusive fashion (22,26,34).

The CD3 protein complex consists of at least five chains: the three structurally related CD3-γ, CD3-δ, CD3-ϵ chains and two disulfide-linked CD3-ζ chains, which probably play an important role in signal transduction from the TcR to the cytoplasm (Figure 2)(10). Northern blot studies on T-ALL cell samples revealed that CD3-δ and CD3-ϵ mRNA was not only detectable in $CD3^+$ T-ALL, but also in all $CD3^-$ T-ALL tested (29,39).This was in line with the presence of CD3-δ and CD3-ϵ proteins in the cytoplasm (CyCD3) of all $CD3^-$ T-ALL (including weak expression in the prothymocytic T-ALL) and the majority of $CD3^+$ T-ALL, as detected by monoclonal antibodies (McAb)(27). Apparently CyCD3 is already expressed in immature T cells, which do not yet bear a TcR-CD3 complex at the cell surface. Identical results were obtained in studies on normal thymocytes (27,40,41). Therefore CyCD3 is an excellent marker for immature normal T cells and their malignant counterparts (Figure 1)(27,41). The completely assembled TcR-CD3 complex is found at the cell surface of mature thymocytes and peripheral T lymphocytes only (27).

The TcR-CD3 complex is expressed by 60-65% of normal thymocytes (42), while only 30-35% of the T-ALL express CD3 on their membrane (Table 1). This discrepancy might be explained by the fact that a total thymocyte suspension includes TdT^+ cortical cells as well as mature $CD3^+$ medullary cells, while T-ALL only represent the TdT^+ T cell population. Since more than 95% of cells in a thymocyte suspension express pan-T cell markers while only 70 to 80% of them express TdT, it can be assumed that 20-30% of the thymocytes probably represent TcR-$CD3^+$ medullary T lymphocytes. This was proven by double immunofluorescence staining, which showed that only 45% of the TdT^+ thymocytes express a TcR-CD3 complex on their cell membrane. This frequency is comparable to the frequency of $CD3^+$ T-ALL (Table 1).

The majority of blood T lymphocytes (85-98%) express TcR-$\alpha\beta$ and a minority (2-15%) express TcR-$\gamma\delta$ (13,14,33). The frequency of normal TcR-$\gamma\delta^+$ cells in the thymus is even lower (0.2-1.0%)(14,43 and Table 1). The frequency of TcR-$\gamma\delta$ expression by TdT^+ thymocytes is extremely low (< 0.1%) when compared to TcR-$\alpha\beta$ expression (43 and Table 1). This is in line with the finding that TcR-$\gamma\delta^+$ cells occur more frequently in the medulla than in the cortex of the thymus (44,45). These data suggest that the development of TcR-$\gamma\delta^+$ cells represents a minor differentiation pathway in the human thymus. However, a relatively large proportion of T-ALL express TcR-$\gamma\delta$ (12%)(46,47 and Table 1). This discrepancy between the frequency of normal TcR-$\gamma\delta^+$/TdT^+ thymocytes and their presumed malignant counterparts in a random

TABLE 1. Expression of the TcR-CD3 complex by normal infant thymocytes and T-ALL.

	CD3 negative	CD3 positive	
		TcR-$\alpha\beta$	TcR-$\gamma\delta$
Infant thymocytes (n=6)	39±9%	60±9%	0.4±0.2%
TdT$^+$ thymocytes[a] (n=3)	56±16%[b]	44±16%[b]	<0.1%[b,c]
T-ALL[d]			
- Campana et al.[e]	26/37	9/37	2/37
- Loiseau et al.[f]	20/33	6/33	7/33
- Van Dongen et al.[g]	22/31	6/31	3/31
Total (percentage)	68/101 (67%)	21/101 (21%)	12/101 (12%)

a. The percentage of TdT$^+$ thymocytes within an infant thymus cell sample is 78±13% (n=11).
b. Data obtained by double immunofluorescence stainings for surface membrane expression of TcR-CD3 molecules and nuclear expression of TdT.
c. Double IF stainings revealed that < 3% of the TcR-$\gamma\delta^+$ thymocytes expressed TdT.
d. The here presented series of T-ALL are claimed to be random. Therefore it is remarkable that the frequency of TcR-$\gamma\delta^+$ T-ALL in the series of Loiseau et al. is more than twice as high than in the series of Campana et al. and Van Dongen et al. However, in a recent report on TcR expression by CD3$^+$ T-ALL cells, it was found that 10 out of 15 T-ALL expressed TcR-$\gamma\delta$, whereas only 5 out of 15 expressed TcR-$\alpha\beta$ (reference 48).
e. Data from reference 46.
f. The expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ in the T-ALL series of Loiseau et al. is deduced from their Southern blot data and data concerning the reactivity of antibodies against TcR molecules (47).
g. Unpublished results.

series of T-ALL may be explained in two ways: either TcR-$\gamma\delta^+$/TdT$^+$ thymocytes are more prone to malignant transformation or a major proportion of TcR-$\gamma\delta^+$ cells develops outside the thymus.

In a recent study differences between T-ALL and T cell lymphoblastic lymphomas (T-LBL), it was found that the majority of CD3$^+$ T-ALL express TcR-$\gamma\delta$ (10 out of 15), while the majority of CD3$^+$ T-LBL express TcR-$\alpha\beta$ (13 out of 15)(48). Based on clinical similarities, T-LBL are generally regarded as lymphomatous variants of T-ALL. However, T-LBL patients have mediastinal tumors (thymic location), pleural effusions and/or lymph node involvement with no or minimal BM involvement, whereas patients with T-ALL present with massive BM and blood invasion (48). This would imply that T-LBL represent more precise malignant counterparts of TdT$^+$ cortical thymocytes than T-ALL and that T-ALL also include malignant counterparts of extrathymic immature T cells.

HUMAN TcR GENES AND THEIR REARRANGEMENT DURING T CELL DIFFERENTIATION

The TcR protein chains consist of a variable (V) domain and a constant (C) domain (Figure 2), which are encoded by separate exons. A functional V domain exon arises during a process of gene rearrangement, which mediates the joining of a particular combination of V, diversity

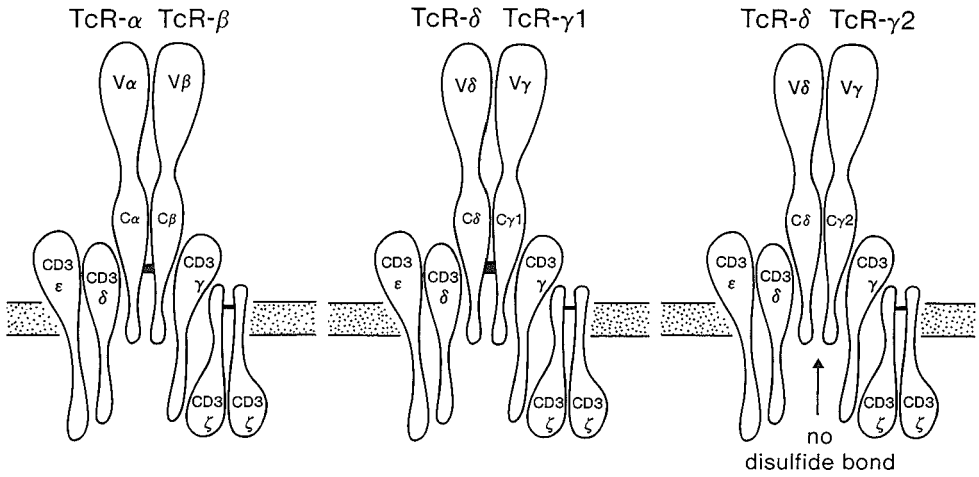


Figure 2. Schematic diagram of the various types of human TcR-CD3 complexes. The TcR molecules consist of two chains, each of which contain a V domain and C domain. In TcR-$\alpha\beta$ and TcR-$\gamma 1\delta$ molecules the two chains are linked via a disulfide bond. This interchain disulfide bond is lacking in TcR-$\gamma\delta$ molecules, in which the TcR-γ chain is derived from the Cγ2 locus (31,66,67).

(D) and joining (J) gene segments in case of the TcR-β and TcR-δ genes or V and J gene segments in case of the TcR-α and TcR-γ genes. The enormous diversity of TcR molecules, needed to recognize a wide variety of antigens, is generated via these gene rearrangement processes.

During the last five years detailed information has become available about the configuration and rearrangement of the four human TcR genes. The germline configuration of the human TcR genes is given in Figure 3. The TcR-α locus contains many different V gene segments, a long stretch of J gene segments and one C gene segment (49,50). The TcR-β locus consists of many different V and two C gene segments, both of which are preceded by one D and six or seven J gene segments (51-53). The TcR-γ locus consists of a restricted number of V gene segments (eight functional and seven pseudo genes) and two C segments (γ1 and γ2), each of which are preceded by two or three J gene segments (30,54-56). Curiously, the TcR-δ locus is situated in between the Vα and Jα gene segments (57-59) and probably consists of only a few V, three D, three J and one C gene segment (60-63). Due to their localization within the TcR-α gene locus, the TcR-δ genes can theoretically draw their V gene segments from the much larger pool of Vα gene segments.

It is obvious that many different Vα-Jα and Vβ-Dβ-Jβ combinations can be obtained, while the combinatorial repertoire of the TcR-γ and TcR-δ genes is restricted due to the limited number of available V, (D) and J gene segments (19). However, the TcR-γ and particularly the TcR-δ genes have an enormous junctional repertoire, i.e. diversity due to insertion of nucleotides (N regions) at the joining sites of the gene segments (15,64,65).

In all TcR-$\alpha\beta$ molecules and TcR-$\gamma 1\delta$ molecules the two protein chains are linked by a disulfide bond (Figure 2). This interchain disulfide bond is lacking in TcR-$\gamma\delta$ molecules, in which

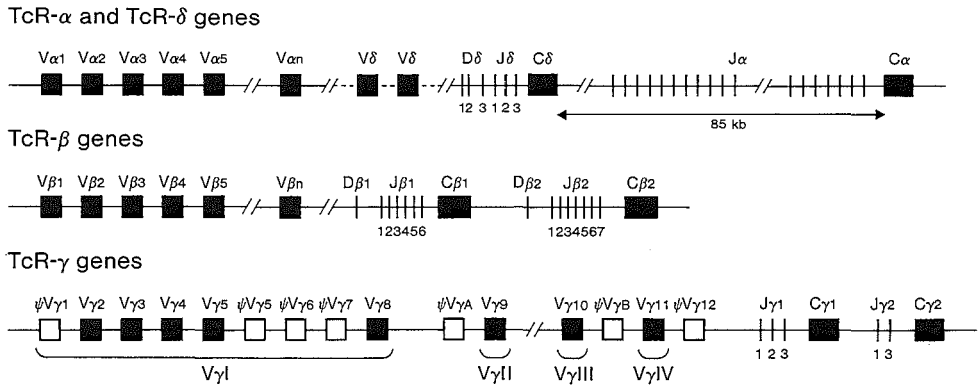


Figure 3. Schematic diagram of the germline configuration of the four human TcR genes. The TcR-α genes consist of many Vα gene segments, a remarkably long stretch of Jα gene segments and one Cα gene segment. The TcR-δ locus is located within the TcR-α gene complex between the Vα and the Jα gene segments and consists of a few Vδ, three Dδ, three Jδ and one Cδ gene segments. The TcR-β genes contain many Vβ and two Cβ gene segments, which are preceded by a Dβ and six or seven Jβ gene segments. The TcR-γ genes consist of two Cγ gene segments, preceded by two or three Jγ gene segments and a restricted number of Vγ gene segments: eight functional genes and seven pseudo-genes (ψ; open boxes). The functional Vγ genes are grouped in four families (VγI, VγII, VγIII and VγIV) based on degrees of sequence homology.

the TcR-γ chain is derived from Cγ2 genes (31,66,67). This is due to the absence of a cysteine residue in the second exon of Cγ2 (66-68). It is not clear whether Cγ1 and Cγ2 encoded TcR-γδ define functionally distinct lineages. It has been suggested that TcR-γ1δT lymphocytes exhibit a higher degree of non-MHC-restricted cytotoxicity than the TcR-γ2δ T lymphocytes (69). Also differences in morphology, cytoskeletal organization and growth characteristics have been found (70).

Hierarchic order of rearrangement and transcription of TcR genes studied in T-ALL

Initial studies concerning TcR-β and TcR-γ in T-ALL revealed that a large part of the $CD3^-$ T-ALL (83-90%) have rearranged TcR-β and TcR-γ genes, suggesting that both genes rearrange early during T cell differentiation (29,39,71-79). Probably the TcR-γ genes are rearranged and transcribed prior to the TcR-β genes, since a few $CD3^-$ T-ALL with rearranged TcR-γ genes but germline TcR-β genes have been reported (46,47,72 and Table 2).

The TcR-α gene configuration is difficult to analyze because of the long stretch of Jα gene segments (49,50). So far only one detailed report on the configuration of TcR-α genes in T-ALL has been published (79), but several papers describe the transcription of rearranged TcR-α genes in T-ALL (29,39,72,74,75,79). These reports indicate that most $CD3^-$ T-ALL have germline TcR-α genes and that rearrangement and transcription of TcR-α genes is mainly found in $CD3^+$ T-ALL. This suggests that the TcR-α genes rearrange late during T cell development.

During the last two years also data concerning the TcR-δ genes have become available (32,46,47,80-83). A few $CD3^-$ T-ALL have germline TcR-β, TcR-γ and TcR-δ genes, but the ma-

TABLE 2. TcR gene rearrangements in CD3$^-$ and CD3$^+$ T-ALL.

	TcR-β genes rearranged	TcR-γ genes rearranged	TcR-δ genes	
			rearranged (R/G or R/R or R/D)	deleted (D/G or D/D)
CD3$^-$ T-ALL[a]				
Hara et al. (79,80)	7/7	7/7	6/7	1/7
Hockett et al. (81)	1/2	1/2	2/2	0/2
Foroni et al. (82)	0/2	0/2	2/2	0/2
Loiseau et al. (47)	15/16	20/20	17/20	3/20
Van Dongen et al. (32)[b]	5/6	5/6	5/6	0/6
Campana et al. (46)	11/14	13/14	12/14	0/14
Total (percentage)	39/47 (83%)	46/51 (90%)	44/51 (86%) 48/51 (94%)	4/51 (8%)
CD3$^+$ T-ALL[a]				
Hara et al. (79,80)	12/12	12/12	7/12	5/12
Hockett et al. (81)	7/8[c]	8/8	3/8	5/8
Foroni et al. (82)	3/3	2/2	2/3	1/3
Loiseau et al. (47)	7/9[c]	13/13	8/13	5/13
Van Dongen et al. (32)[b]	11/11	11/11	7/11	4/11
Campana et al. (46)	9/9	9/9	4/9	5/9
Total (percentage)	49/52 (94%)	55/55 (100%)	31/56 (55%) 56/56 (100%)	25/56 (45%)

a. The here presented series of CD3$^-$ T-ALL and CD3$^+$ T-ALL may not be representative for all T-ALL.
b. The data of this series of T-ALL were partly published in reference 32.
c. The three CD3$^+$ T-ALL with germline TcR-β genes appeared to express TcR-$\gamma\delta$ (47,81).

jority of the CD3$^-$ T-ALL (~95%) have rearranged one or both TcR-δ alleles, while some have deleted one or both alleles (32,46,47,80-82). Also several CD3$^-$ T-ALL with germline TcR-β and/or TcR-γ genes appeared to have rearranged TcR-δ genes (Table 2) (81,82). These data indicate that TcR-δ genes rearrange early during T cell differentiation, probably prior to other TcR genes. CD3$^-$ T-ALL with rearranged TcR-δ genes generally only contain immature TcR-δ transcripts (i.e. "truncated" transcripts, lacking V gene sequences) (32,80,81), whereas mature TcR-δ transcripts are found in TcR-$\gamma\delta^+$ T-ALL (32,81).

In all CD3$^+$ T-ALL rearrangements and/or deletions of the TcR-δ gene locus were found (Table 2). Deletion of one or both TcR-δ alleles is associated with rearrangement of the TcR-α genes (80). This is in line with the location of the TcR-δ genes within the TcR-α locus in between the Vα and Jα gene segments (57-59).

As expected, all TcR-$\gamma\delta^+$ T-ALL have rearranged at least one TcR-γ allele and at least one TcR-δ allele. In one TcR-$\gamma\delta^+$ T-ALL we found one rearranged and one deleted TcR-δ allele, suggesting that a TcR-α gene rearrangement had occurred on one allele (Table 3). A large part of the TcR-$\gamma\delta^+$ T-ALL (~80%) also have rearranged TcR-β genes (Table 3). Detailed analyses of the TcR-γ and TcR-β genes in TcR-$\gamma\delta^+$ T-ALL and TcR-$\gamma\delta^+$ T cell clones have revealed that

TABLE 3. TcR gene rearrangements in TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T-ALL.

	TcR-β genes rearranged	TcR-γ genes rearranged	TcR-δ genes		
			R/G or R/R	R/D	D/G or D/D
TcR-$\alpha\beta^+$ T-ALL					
Hockett et al. (81)	7/7	7/7	0/7	2/7	5/7
Loiseau et al. (47)[a]	5/5	6/6	0/6	1/6	5/6
Van Dongen et al. (32)[b]	4/4	4/4	0/4	0/4	4/4
Campana et al. (46)	7/7	7/7	0/7	2/7	5/7
Total	23/23	24/24	0/24	5/24	19/24
(percentage)	(100%)	(100%)	(0%)	(21%)	(79%)
TcR-$\gamma\delta^+$ T-ALL					
Hockett et al. (81)	0/1	1/1	1/1	0/1	0/1
Loiseau et al. (47)[a]	2/4	7/7	7/7	0/7	0/7
Van Dongen et al. (32)[b]	7/7	7/7	6/7	1/7	0/7
Campana et al. (46)	2/2	2/2	2/2	0/2	0/2
Total	11/14	17/17	16/17	1/17	0/17
(percentage)	(79%)	(100%)	(94%)	(6%)	(0%)

a. The expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ in the T-ALL series of Loiseau et al. is deduced from their Southern blot data and data concerning the reactivity of antibodies against TcR molecules (47).
b. The data of this series of T-ALL were partly published in reference 32.

TcR-$\gamma 1\delta^+$ T cells generally have germline TcR-β genes or incomplete (Dβ-Jβ) rearrangements, whereas TcR-$\gamma 2\delta^+$ T cells generally have incomplete (D-J) or complete (V-D-J) TcR-β gene rearrangements (31,84). Transcripts of these rearranged TcR-β genes are often found in TcR-$\gamma\delta^+$ cells (31,32,84) and even TcR-β proteins may be present in the cytoplasm (85).

All TcR-$\alpha\beta^+$ T-ALL have rearranged one or both TcR-β genes. In ~80% of them both TcR-δgene alleles are deleted and in ~20% one TcR-δ allele is deleted, which is in line with rearrangements of the TcR-α genes (Table 3) (32,46,47,81). The TcR-γ genes are rearranged in all TcR-$\alpha\beta^+$ T-ALL (Table 3) and in a few of them also low amounts of TcR-γ transcripts are found (32,81).

Apparently TcR gene rearrangements and even TcR gene expression overlap in $\gamma\delta$ and $\alpha\beta$ cells without leading to aberrant TcR protein expression. There obviously exist exclusion mechanisms to avoid dual receptor expression. Deletion of TcR-δ genes in TcR-$\alpha\beta^+$ cells represents one of such mechanisms (see below).

Based on the combined T-ALL data we have previously postulated a linear T cell differentiation scheme (Figure 4). The TcR-δ genes rearrange first. Subsequently, the TcR-γ genes rearrange to the Cγ1 locus prior to or coinciding with D-J joining of the TcR-β genes, potentially followed by rearrangement to the Cγ2 locus and V-D-J joining of the TcR-β genes (31). If the TcR-γ and TcR-δ gene rearrangements are functional, TcR-$\gamma 1\delta^+$ T cells or TcR-$\gamma 2\delta^+$ T cells

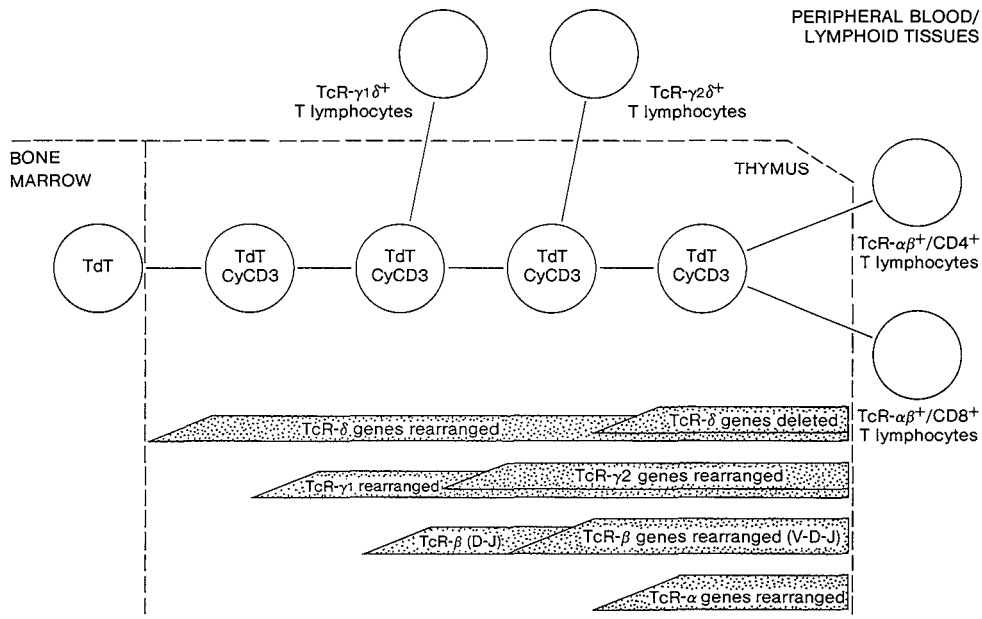


Figure 4. Hypothetical scheme of human T cell differentiation with a linear sequentially ordered rearrangement of TcR genes (31). The horizontal bars summarize the hierarchic order of TcR gene rearrangement as deduced from studies on T-ALL and human TcR-$\gamma\delta^+$ T cell clones. The dotted lines mark the tissue compartments, where the cells in the various T cell differentiation stages are found.

may develop. If one of the rearrangements is not functional, the T cell may complete the TcR-β gene rearrangement together with deletion of the TcR-δ genes and rear-rangement of the TcR-α genes. If these rearrangements are functional, TcR-$\alpha\beta^+$ T cells may develop. This linear T cell differentiation pathway is supported by the presence of rearranged TcR-γ gene (generally to the γ2 locus) in all $CD3^+$ T-ALL (32,46,71,74,75,78), of which a large part probably expresses TcR-$\alpha\beta$. Most of the TcR-γ gene transcripts in TcR-$\alpha\beta^+$ cells are nonfunctional (87-89).

The linear sequentially ordered rearrangement of TcR genes is also in line with data concerning murine T cell ontogeny, which indicate that rearrangement of the TcR-δ genes occurs at least as early as day 14 in fetal thymocytes (90-92), at the same time or before rearrangement and transcription of the TcR-γ genes (91,93-95). TcR-β genes start to rearrange from day 14 on, resulting in fully rearranged genes and mature 1.3 kb transcripts two days later (93-97). Rearrangement and transcription of the TcR-α genes occurs from day 17 on (94,95). TcR-$\gamma\delta$ is indeed expressed by day-14 and day-15 fetal thymocytes, whereas the first TcR-$\alpha\beta^+$ thymocytes can be detected two days later (98-101).

New hypothetical scheme of T cell differentiation

Although most if not all T-ALL fit in the postulated linear T cell differentiation scheme (Figure

4), recent findings suggest that the $\gamma\delta$ and $\alpha\beta$ differentiation pathways are separated in an early stage and that cells in the $\alpha\beta$ pathway are not necessarily derived from cells with unsuccessful rearrangement of their TcR-γ and/or TcR-δ genes. This early divergence of $\alpha\beta$ and $\gamma\delta$ pathways is probably regulated via a combination of silencers of the TcR-α gene enhancer and rearrangement/deletion mechanisms in the TcR-α/δ gene locus. We will present a new hypothetical differentiation scheme based on new data in the literature as well as our own T-ALL data.

1. TEA-Cα transcripts.

In immature T cells a curious 2.0 kilobase (kb) mRNA with Cα sequences was found, which did not contain a Vα gene segment but a tandemly repetitive motif called "T early α" (TEA) (102). The TEA-Cα transcripts are sterile, i.e. not translatable into protein (102). The TEA gene segment is located in between the TcR-δ genes (~10 kb 3′ of Cδ) and the Jα genes (~75 kb 5′ of Cα) and is not rearranged in the immature T cells which express the TEA-Cα transcripts (Figure 5)(81,102). This implies that the 2.0 kb TEA-Cα transcript is a splicing product of a long (75-80 kb) precursor mRNA. The TEA-Cα transcript has been found in immature CD3$^-$ T-ALL (81) and human fetal thymocytes (102), but also in a TcR-$\gamma\delta^+$ T-ALL (74,81,102). The TEA gene segment remains in germline configuration in immature T cells as well as in TcR-$\gamma\delta^+$ T cells, but is deleted in TcR-$\alpha\beta^+$ cells, in line with its genomic location (102). It has been suggested that TEA-Cα transcription may play a regulatory role in opening the Jα locus to obtain TcR-δ deletion and subsequent Vα-Jα rearrangement (81).

2. TcR-δ deleting elements.

The human TcR-δ locus is flanked by δ deleting elements that undergo preferential rearrangement in the thymus, resulting in deletion of internal TcR-δ gene segments (103,104). One deleting element is located ~12 kb 3′ of Cδ, just 3′ of the TEA gene segment and just 5′ of the Jα gene segments (Figure 5). This deleting element is called ψJα, because of its high homology to Jα gene segments and a heptamer-nonamer sequence at the 5′ side, which represents a specific joining sequence for gene rearrangement (103). It is likely to be a pseudogene since the Jα consensus sequence is incomplete (103). The second deleting element is called δREC and is located 5′ of the TcR-δ locus, but 3′ of the Vδ1 gene; its precise position is not yet known (103). In the murine TcR-δ locus at least three δREC gene segments are present (104,105), which may also be the case in man. The known human δREC gene segment is flanked by a consensus heptamer-nonamer sequence at its 3′ side, which permits rearrangement to ψJα with concomitant deletion of the TcR-δ locus. The δREC-ψJα rearrangement occurs at high frequency in the developing thymus (103,104) and has been found in an immature T-ALL (106) as well as some T cell lines (103). The TcR-δ deleting rearrangement event probably represents an important mechanism in separating the $\alpha\beta$ and $\gamma\delta$ lineages, because cells undergoing δREC-ψJα rearrangement on both alleles will not be able to express TcR-$\gamma\delta$. However, certain functional Vδ-Dδ rearrangements on both alleles will delete the δREC gene segment which may hamper differentiation along the $\alpha\beta$ pathway.

3. Circular excision products.

The joining of gene segments during rearrangement probably occurs via a process of looping-out and excision of the intervening sequences (107,108). It is generally assumed that

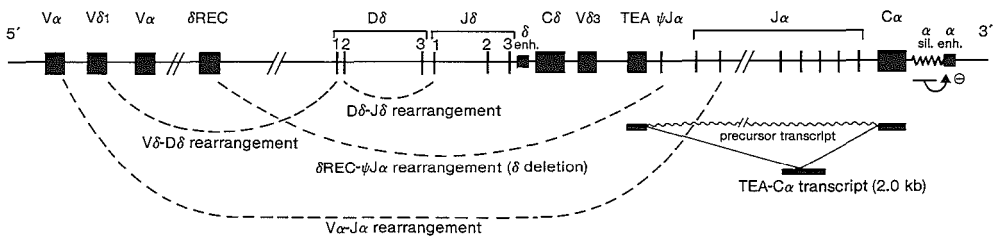


Figure 5. Schematic diagram of the human TcR-α/δ locus. The various gene segments are presented: TcR-δ deleting elements (δREC and ψJα), "T early α" (TEA) gene segment, TcR-δ gene enhancer (δ enh.) and the TcR-α gene enhancer (α enh.) with its flanking silencer sequences (α sil.). The possible gene rearrangements and the TEA-Cα transcription are indicated (see text).

such gene rearrangements start with the back-to-back fusion of the heptamer-nonamer joining sequences, which flank the involved gene segments (i.e. the 3′ joining sequences of the upstream gene segment and the 5′ joining sequences of the downstream gene segment)(107). With joining of two TcR gene segments a circular excision product is formed, which contains a head-to-head structure of the two heptamer-nonamer sequences as well as all intervening TcR gene sequences (107,108). Such excised circles could indeed be isolated from murine thymocytes and sequences of all four murine TcR genes have been identified in these small circular excision products (105,109-113).

Detailed studies on the occurrence of TcR-α and TcR-δ gene sequences in circular excision products have proven that at least the following types of rearrangement events can occur in the TcR-α/δ locus:

- Dδ1-Dδ2 joining (105);
- incomplete TcR-δ gene rearrangements (V-Dδ joining) can be replaced by Vδ-Jδ rearrangements (112);
- non-functional complete TcR-δ gene rearrangements (V-D-D-J) can be deleted (105);
- germline TcR-δ sequences (Dδ-Jδ region) can be deleted (113);
- δREC-ψJα rearrangements can be followed by Vα-Jα rearrangements (105,111);
- Vα-Jα rearrangements can be followed by new Vα-Jα rearrangements of an upstream Vα and a downstream Jα gene segment (111,112);
- the head to head joints of the heptamer-nonamer joining sequences (reciprocal joints) in circular excision products of rearranged TcR-δ genes are often imprecise with insertion or deletion of several bases (105), while virtually all excision products of TcR-α gene rearrangements contain precisely fused heptamer sequences (109,111,112).

These combined data indicate that progressive rearrangements can occur in the TcR-α/δ locus on the same chromosome. This includes the deletion of germline TcR-δ genes or non-functionally rearranged TcR-δ genes, followed by TcR-α gene rearrangements.

4. Silencer of the TcR-α gene enhancer.

The enhancer for transcription of the murine TcR-α gene is located ~3 kb downstream of the Cα gene segment; it is assumed that the location in the human genome is comparable (Figure 5)(114). The unique structure of the TcR-α locus suggests that the enhancer can act

over ~70 kb, up to the most 5′ located Jα gene segment. Transfection experiments have demonstrated that the TcR-α enhancer is indeed very powerful and that its activity is T cell specific (114). This lineage specificity of the TcR-α enhancer is achieved through the activation of multiple negative *cis*-acting elements, which silence the enhancer in non-T cells as well as in TcR-$\gamma\delta^+$ cells, but not in TcR-$\alpha\beta^+$ cells (115). These silencers are located in between the Cα gene segment and the α enhancer (115). Since germline transcription is generally thought to precede rearrangement, the silencing of the TcR-α locus in cells of the γδ lineage would explain why the Jα genes are not targets of rearrangements in TcR-$\gamma\delta^+$ cells.

5. TcR-δ enhancer elements.

Two transcription enhancing elements have been identified in the human TcR-δ gene locus in between the Jδ3 and Cδ gene segments (Figure 5)(116). This localization implies that the enhancer remains undeleted during all types of TcR-δ gene rearrangements. Transfection experiments with one of the TcR-δ gene enhancers demonstrated activity in both TcR-$\gamma\delta^+$ cells and TcR-$\alpha\beta^+$ cells, but not in B cells or fibroblasts (116). This indicates that this enhancer is T cell specific, but not restricted to cells of the γδ pathway.

6. TcR-γ gene silencer.

Recent studies in transgenic mice suggest that transcription of functionally rearranged TcR-γ genes in TcR-$\alpha\beta^+$ cells is repressed via silencer sequences (101,117,118). In transgenic mice constructed with a "short" TcR-γ gene clone the transgene transcripts accumulate abundantly, which is associated with a severe blockage in the generation of TcR-$\alpha\beta^+$ cells, while TcR-γδ cells are present in relatively high frequencies (21,117). However, in transgenic mice constructed with a "long" TcR-γ gene clone no accumulation of transgene transcripts occurs, which is accompanied with an apparently normal generation of TcR-$\alpha\beta^+$ T cells. This data suggests that the TcR-γ gene flanking sequences (101,118) contain *cis*-acting silencer elements, which are absent in the "short", but present in the "long" TcR-γ transgenes (101,117,118).

The investigators hypothesized that T cell precursors can be committed to generating TcR-$\alpha\beta^+$ cells, independently of the rearrangement status of their TcR-γ genes and that this commitment involves repression of TcR-γ gene transcription through interaction with a *cis*-regulatory DNA element (a silencer)(101,117). They further suggested that this γ-silencer model applies to the separation of αβ and γδ cell lineages (101,117). However, the data cannot indicate whether this separation occurs in an early or late stage of T cell differentiation. Furthermore, the repression of TcR-γ gene transcription in cells of the αβ lineage is only necessary, if the TcR-γ genes are rearranged functionally. Although virtually all TcR-$\alpha\beta^+$ cells have rearranged TcR-γ genes, in most cases these rearrangements appear to be non functional (87-89)

Based on the above data, we have postulated a new T cell differentiation scheme in which an early separation of the αβ pathway and γδ pathway is depicted (Figure 6). The complex organization of the TcR-α/δ locus necessitates differential accessibility of this gene locus in the two different pathways (Figure 5). Therefore it is suggested that the activity of the silencer of the TcR-α genes is one of the main regulatory mechanisms in the new hypothetical differentiation scheme.

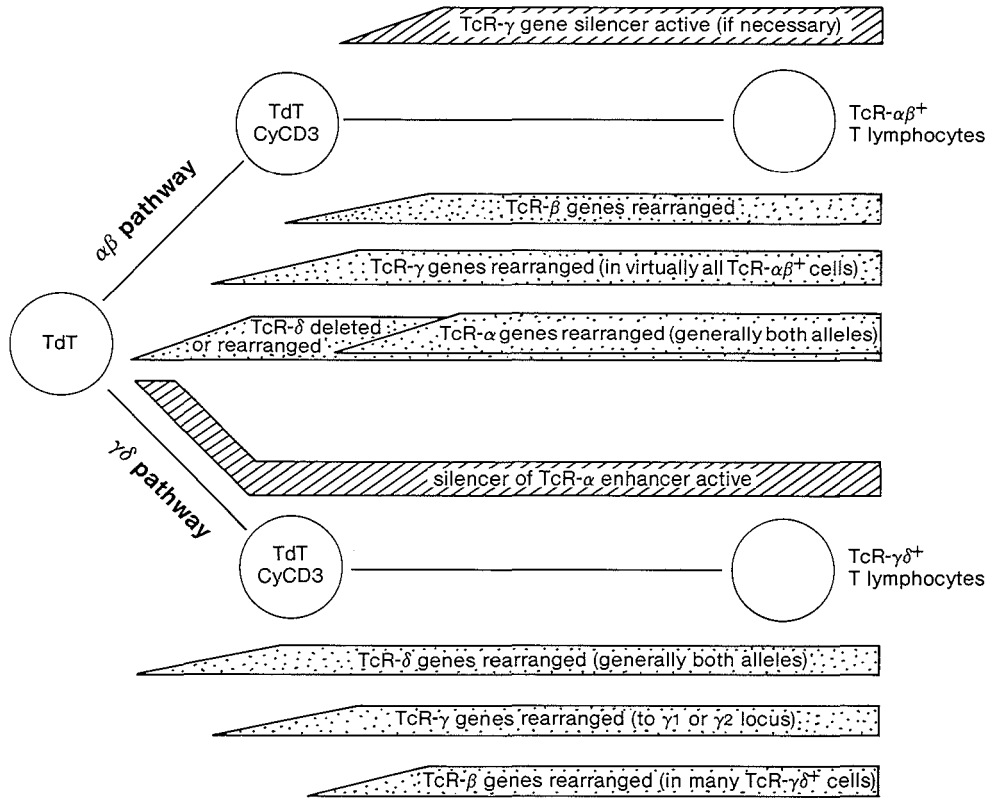


Figure 6. Hypothetical scheme of human T cell differentiation with separated $\alpha\beta$ and $\gamma\delta$ pathways. The dotted bars summarize the hierarchic order of TcR gene rearrangements for each pathway. The shaded bars represent the activity of the TcR-α gene silencer in the $\gamma\delta$ pathway and the TcR-γ gene silencer in the $\alpha\beta$ pathway. It is assumed that the TcR-α gene silencer plays a central role in the early separation of the $\alpha\beta$ pathway and $\gamma\delta$ pathway (115). As long as the TcR-α silencer remains active, the rearrangements in the TcR-α/δ locus are restricted to the TcR-δ genes. If the α silencer is inactivated, rearrangements in the TcR-α locus will occur (see text). The TcR-γ silencer is necessary to repress transcription of possibly functionally rearranged TcR-γ genes in TcR-$\alpha\beta^+$ cells.

One of the earliest events during T cell differentiation probably is the T cell specific activation of the TcR-δ enhancer elements, resulting in transcription-mediated opening of the TcR-δ locus in all immature T cells (116). If the α silencer remains active, only rearrangements in the TcR-δ genes can occur (115), immediately followed by TcR-γ gene rearrangements. In many cells of the $\gamma\delta$ pathway also rearrangements in the TcR-β genes will occur. If the rearrangements of the TcR-δ and TcR-γ genes are functional, this may result in TcR-$\gamma\delta^+$ cells. The TcR-α genes in TcR-$\gamma\delta^+$ cells remain in germline configuration due to the continuous activity of the α silencer (115).

If the α-silencer becomes inactivated, the TcR-α enhancer can induce transcription-mediated opening of the TcR-α locus (115), e.g. via TEA-Cα transcription (81). This transcription opens the ψJα gene segment, while the structure of the δREC gene segment was already opened via

the early activation of the TcR-δ gene enhancer (116). Subsequently δREC-ψJα rearrangement may occur (103,104), resulting in the deletion of germline TcR-δ gene sequences (113) or incompletely rearranged TcR-δ gene sequences. Even completely rearranged TcR-δ genes can be deleted (105). As a consequence of such deletions the Vα gene segments are positioned in closer proximity to the TcR-α enhancer, which may facilitate the subsequent Vα-Jα rearrangements (105,111). In the mean time also the TcR-γ and TcR-β genes are rearranged. Functional TcR-α and TcR-β gene rearrangements may result in TcR-$\alpha\beta^+$ cells in which an activated γ-silencer represses transcription of a possibly functionally rearranged TcR-γ gene (101,117,118).

The hierarchic order of TcR gene rearrangements does not essentially differ between the linear differentiation scheme (Figure 4) and the differentiation scheme with separate $\alpha\beta$ and $\gamma\delta$ pathways (Figure 6). The main difference between the two schemes is the postulated differential accessibility of the complex TcR-α/δ locus (Figure 5), which is easy to fit into a two-lineage scheme, but difficult into a linear scheme. This emphasizes the central role of the TcR-α/δ locus in T cell differentiation.

The T-ALL data fit into the proposed two-lineage T cell differentiation scheme. In most TcR-$\alpha\beta^+$ T-ALL (~80%) both TcR-δ gene alleles are deleted, but in some of them one rearranged TcR-δ gene allele is present (Table 3). Whether such a rearranged TcR-δ gene is transcribed in a TcR-$\alpha\beta^+$ cell, has not been reported. Virtually all TcR-$\alpha\beta^+$ cells have rearranged TcR-γ genes (Table 3). However, TcR-γ gene transcripts are generally absent or occur in low amounts in TcR-$\alpha\beta^+$ cells (32,81), which is in line with the activation of a γ-silencer in cells of the $\alpha\beta$ lineage (101,117,118).

In virtually all TcR-$\gamma\delta^+$ cells both TcR-δ gene alleles are rearranged (32,46,47,81). Therefore they lack rearrangement and transcription of TcR-α genes, which is in line with an activated silencer of the TcR-α enhancer in TcR-$\gamma\delta^+$ cells (115). Expression of rearranged TcR-β genes in TcR-$\gamma\delta^+$ cells appears to be possible (31,32,84,85). Apparently no TcR-β gene silencer exists. This may sometimes lead to aberrant TcR expression such as the recently described expression of TcR-$\beta\delta$ by the DND41 T cell line (119). However, in most T cells such aberrant TcR expression will not occur. Even transfection of functional TcR-α gene sequences in TcR-$\gamma\delta^+$ T cells with functional TcR-β genes resulted in the expression of TcR-$\alpha\beta$ and/or TcR-$\gamma\delta$ molecules, but no aberrant TcR molecules were formed (120,121). This indicates that a preferential association of TcR-α and β chains and TcR-γ and δ chains occurs.

THYMUS DEPENDENCY OF THE DEVELOPMENT OF TcR-$\gamma\delta^+$ CELLS

The far majority of mature TcR-$\alpha\beta^+$ cells express CD4 or CD8 molecules, which play a supportive role in MHC restricted antigen recognition (9,15). In the interaction between TcR-$\alpha\beta^+$ T lymphocytes and antigen presenting cells, the CD4 and CD8 molecules associate with MHC-class II and class I molecules, respectively. It is generally assumed that TcR-$\alpha\beta^+$ cells are positively selected for the capability to interact with self MHC molecules in the thymus and that the CD4 and CD8 molecules are involved in this process (9,15,122).

Therefore it is intriguing that most TcR-$\gamma\delta^+$ cells do not express CD4 or CD8 molecules (11-

14,33). This may suggest that TcR-$\gamma\delta^+$ cells are not subject to such thymic selection. It should however be emphasized that in peripheral blood 0,5-2% of TcR-$\gamma\delta^+$ cells express the CD4 molecule (14) and 5-10% the CD8 molecule (Table 4). Perhaps these TcR-$\gamma\delta^+$ lymphocytes have undergone thymic selection, while the $CD4^-/CD8^-$ $\gamma\delta$ cells may be derived from an extrathymic differentiation pathway or exported from the thymus before thymus selection.

As indicated in one of the previous sections, there is evidence that at least a part of theTcR-$\gamma\delta^+$ cells may develop along an extrathymic pathway. First, the frequency of TcR-$\gamma\delta^+$ cells in the thymus is low (Table 1). Second, virtually no TdT^+ $\gamma\delta$ cells are detectable in the thymus, while a large part of the TdT^+ thymocytes express TcR-$\alpha\beta$ (Table 1). One could argue that TcR-$\gamma\delta^+$ cells do not need TdT activity during their development, but the contrary is probably true, because the TdT-mediated junctional diversity (36-38) is more extensive in TcR-$\gamma\delta^+$ cells than in TcR-$\alpha\beta^+$ cells (15,64,65). Third, the frequency of TcR-$\gamma\delta^+$ cells in the medulla is higher than in the cortex of the thymus (44,45). Fourth, the frequency of TcR-$\gamma\delta$ expression by T-LBL is much lower than by T-ALL, although T-LBL represent more precise malignant counterparts of cortical thymocytes (48).

Evidence for extrathymic development of TcR-$\gamma\delta^+$ cells originally came from studies on TcR-γ and TcR-δ gene transcripts in athymic (nude) mice (123,124). However, recent studies in normal mice indicate that the TcR-$\gamma\delta^+$ cells in murine epidermis and epithelia of reproductive organs have emerged from the fetal thymus in spatially distinct waves (100,101,125-129). TcR-$\gamma\delta^+$ cells are indeed absent at these epithelial sites in nude mice (101,130), but present in spleen (123,124,131), lymph nodes (18), and intestine (101). Also in man no predominance of TcR-$\gamma\delta^+$ cells in epidermis and epithelia of reproductive organs has been found (44,45,132). Therefore, the various TcR-$\gamma\delta^+$ cell lineages in mice may have different environmental requirements for development (101,131), whereas in man there is no evidence yet for direct

TABLE 4. Expression of CD4 and CD8 molecules by TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T lymphocytes in blood from healthy individuals

	TcR-$\alpha\beta^+$T lymphocytes[a]		TcR-$\gamma\delta^+$ T lymphocytes[b]	
	$CD4^+$	$CD8^+$	$CD4^+$	$CD8^+$
Children: 0-2 y	65-85% (n=35)	15-35%	1±1% (n=16)	10±7%
Children: 2-16 y	50-70% (n=62)	30-50%	0.5±0.5% (n=17)	5±4%
Adults	50-75% (n=16)	25-50%	2±5% (n=16)	9±7%

a. Normal values for expression of CD4 and CD8 molecules by TcR-$\alpha\beta^+$ T lymphocytes, as determined in the immunodiagnostic laboratory of the Department of Immunology, University Hospital Dijkzigt/ Erasmus University, Rotterdam. The numbers of healthy individuals tested per age group are given in parentheses.

b. Expression of CD4 and CD8 molecules by TcR-$\gamma\delta^+$ T lymphocytes as determined by double immunofluorescence stainings (25,33). The numbers of healthy individuals tested per age group are given in parentheses.

involvement of the thymus in differentiation of TcR-$\gamma\delta$ cells.

To study the situation in man, we have enumerated TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T lymphocytes in blood samples from eight infants with DiGeorge anomaly (DGA) (33). DGA is characterized by facial, cardiac, parathyroid and thymic defects, due to malformations or disruptions of the pharyngeal arches and pouches from which these organs develop (133-136). The degree of thymus hypoplasia or aplasia in DGA is assumed to determine the degree of T cell immunodeficiency (i.e. from partial to complete) (134,136). The eight patients represented the broad spectrum from partial to complete DGA with respect to their T cell immunodeficiency. Seven patients had partial DGA with variable degrees of T cell deficiency and one patient had complete DGA with extremely low numbers of blood T lymphocytes. To obtain normal values for the expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ by peripheral T lymphocytes, we collected blood samples from hundred-eleven healthy children in different age groups.

In the healthy children the majority of the $CD3^+$ T lymphocytes expressed TcR-$\alpha\beta$, while a minority expressed TcR-$\gamma\delta$ (Figure 7). In the seven infants with partial DGA the decrease in T lymphocytes mainly involved the TcR-$\alpha\beta^+$ cells, while the absolute numbers of TcR-$\gamma\delta^+$ cells were much less affected. In the one patient with complete DGA the great majority of the few remaining $CD3^+$ T lymphocytes expressed TcR-$\gamma\delta$, while virtually no TcR-$\alpha\beta^+$ T lymphocytes were found (Figure 7). Interestingly, the absolute number of TcR-$\gamma\delta^+$ cells in the blood of this complete DGA patient was just below the normal range for age (33). These data indicate that the T cell deficiency in DGA patients is mainly restricted to the TcR-$\alpha\beta^+$ T lymphocytes.

It is generally assumed that the variable degree of T cell immunodeficiency in DGA is directly related to the degree of thymus hypoplasia or aplasia, since the thymic defect in DGA involves thymic mass rather than a dysplastic process, i.e. a quantitative instead of a qualitative defect (134,136). Our data indicate that the development of TcR-$\alpha\beta^+$ cells is severely hampered by decrease in thymic epithelium, while the TcR-$\gamma\delta$ lineage is significantly less affected. This suggests that TcR-$\gamma\delta^+$ T lymphocytes in man can develop via an extrathymic differentiation pathway.

It is remarkable that in healthy children the absolute numbers of T lymphocytes gradually decrease from approximately 4×10^9/l in early infancy to approximately 1.5×10^9/l in adolescence. This decrease only concerns TcR-$\alpha\beta^+$ cells, since the absolute numbers of TcR-$\gamma\delta^+$ cells remain stable (Figure 7). During aging from infancy to adolescence also a prominent involution of the thymus occurs (137,138). This coincidence of thymic involution and selective decrease of TcR-$\alpha\beta^+$ cells during aging of children is in line with the relationship between thymic mass and the absolute numbers of TcR-$\alpha\beta^+$ cells, as found in DGA patients.

All data together indicate that at least a part of the human TcR-$\gamma\delta^+$ cells are derived from an extrathymic differentiation pathway. Given that human TcR-$\gamma\delta^+$ cells isolated from peripheral blood display extensive diversity in the junctional region of their TcR-δ genes (61-63,139), this would imply that TdT is probably also active during development of TcR-$\gamma\delta^+$ cells outside the thymus. Except for low frequencies of $CD2^+/TdT^+$ cells and $CD7^+/TdT^+$ cells, no TdT^+ T cells (e.g. $CD3^+/TdT^+$ cells) have been identified outside the thymus so far (24,140-142). However, the putative extrathymic development of TcR-$\gamma\delta^+$ cells may occur at specific (as yet undiscovered) locations.

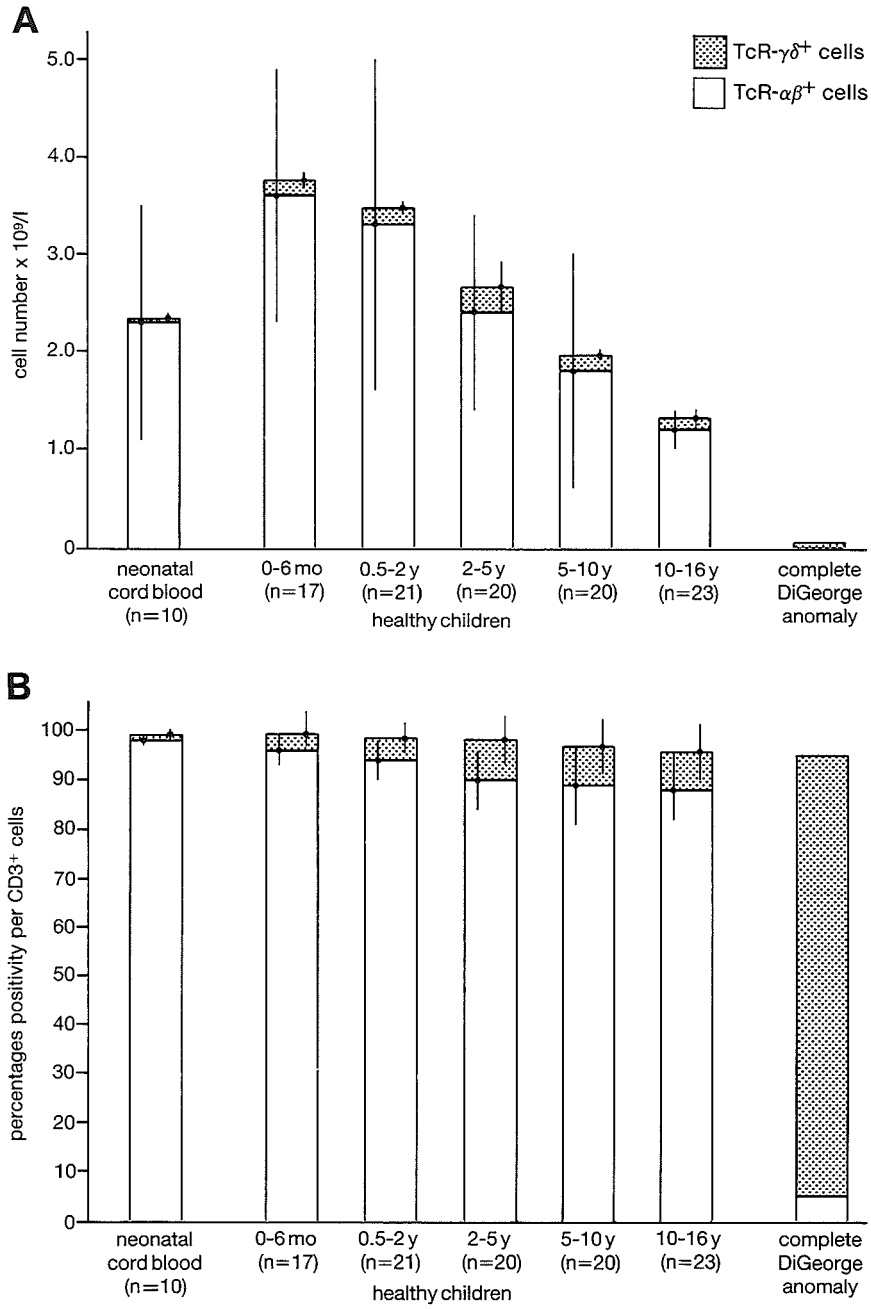


Figure 7. Expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ by $CD3^+$ T lymphocytes in neonatal cord blood, blood from healthy children (five different age groups) and the patient with complete DGA (see reference 33). The analyses were performed by use of TcR/CD3 double immunofluorescence stainings as described (25,33). **A**: absolute numbers of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T lymphocytes. **B**: relative distribution of TcR-$\alpha\beta$ and TcR$\gamma\delta$ expression within the $CD3^+$ T lymphocyte population. The vertical lines in the bars represent the SD values.

CONCLUSION AND SPECULATIONS

Our T-ALL studies indicate that during T cell differentiation a hierarchic order of TcR gene rearrangements exists. Based on these data as well as recent data from the literature we have adapted our linear T cell differentiation scheme to a scheme with an early divergence of the $\alpha\beta$ and $\gamma\delta$ pathway. This early divergence is probably mediated via rearrangement/deletion processes in the TcR-α/δ locus, which are dependent on the activity of the silencer of the TcR-α gene enhancer (Figures 5 and 6).

While the $\alpha\beta$ pathway is assumed to be strictly thymus-dependent, we have strong evidence that at least a part of the $\gamma\delta$ pathway is not. This is based on our immunophenotypic studies of normal thymus and blood samples (Table 1 and Figure 7) as well as on analyses of blood samples from athymic patients, i.e. children with DGA (Figure 7).

Mechanisms of thymic negative and positive selection play a crucial role in the generation of TcR-$\alpha\beta$ cell repertoire and influence the subsequent expression of CD4 and CD8 molecules (143,144). It is not known whether similar thymic selection processes also influence the repertoire of TcR-$\gamma\delta^+$ cells. Concerning the thymus-dependency of T cell differentiation we would like to speculate that in man migration of prothymocytes into the thymus and interaction with thymic epithelial cells will switch off the silencer of the TcR-α gene enhancer. In this way the majority of the thymic T cells are induced to develop along the $\alpha\beta$ pathway, whereas only a minority escape the down regulation of the silencer and thereby develop along the $\gamma\delta$ pathway. This hypothesis would also explain why extrathymic T cell development (as in DGA patients) mainly results in TcR-$\gamma\delta^+$ cells and not in TcR-$\alpha\beta^+$ cells, if one assumes that extrathymic decrease of the activity of the α silencer is an inefficient process. The relatively high frequency of TcR-$\gamma\delta^+$ cells in the thymus during early murine ontogeny (98-101) may be explained by immaturity of the thymic epithelium.

ACKNOWLEDGMENTS. We thank Professor Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Mr. T.M. van Os (Department of Immunology, Erasmus University, Rotterdam) and Mr. C. van Dijk (Interpoint, Hapert) for preparation of the figures and Ms. H.J. de Jager for typing the manuscript.

REFERENCES

1. Basch RS, Kadish JL. Hematopoietic thymocyte precursors II: properties of precursors. J Exp Med 1977; 145:405-19.
2. Lepault F, Weissman IL. An *in vivo* assay for thymus-homing bone marrow cells. Nature 1981;293:151-4.
3. Boersma W, Betel I, Daculsi R, Van der Westen G. Post-irradiation thymocyte regeneration after bone marrow transplantation I: regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet 1981;14:179-96.
4. Ezine S, Weissman IL, Rouse RV. Bone marrow cells give rise to distinct cell clones within the thymus. Nature 1984;309:629-31.
5. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
6. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
7. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.

8. Royer HD, Reinherz EL. T lymphocytes: ontogeny, function, and relevance to clinical disorders. N Engl J Med 1987;317:1136-42.
9. Marrack P, Kappler J. The T cell receptor. Science 1987;238:1073-9.
10. Clevers H, Alarcon B, Wileman T, Terhorst C. The T cell receptor/CD3 complex: a dynamic protein ensemble. Annu Rev Immunol 1988;6:629-62.
11. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
12. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325:683-8.
13. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
14. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.
15. Davis MM, Bjorkman PJ. T-cell antigen receptor genes and T-cell recognition. Nature 1988;334:395-402 and corrigenda 1988;335:744.
16. Littman DR. The structure of the CD4 and CD8 genes. Annu Rev Immunol 1987;5:561-84.
17. Parnes JR. Molecular biology and function of CD4 and CD8. Adv Immunol 1989;44:265-311.
18. Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of $\gamma\delta$ receptor-bearing T lymphocytes. Nature 1987;330:262-4.
19. Strominger JL. The $\gamma\delta$ T cell receptor and class Ib MHC-related proteins: enigmatic molecules of immune recognition. Cell 1989;57:895-8.
20. Bonneville M, Ito K, Krecko EG, Itohara S, Kappes D, Ishida I, Kanagawa O, Janeway CA jr, Murphy DB, Tonegawa S. Recognition of a self major histocompatibility complex TL region product by $\gamma\delta$ T-cell receptors. Proc Natl Acad Sci USA 1989;86:5928-32 .
21. Dent AL, Matis LA, Hooshmand F, Widacki SM, Bluestone JA, Hedrick SM. Self-reactive $\gamma\delta$ T cells are eliminated in the thymus. Nature 1990;343:714-9.
22. Van Dongen JJM, Hooijkaas H, Hählen K, Van Zanen GE. The small subpopulation of T cell marker$^+$/TdT$^+$ cells in the human bone marrow may represent prothymocytes. Adv Exp Med Biol 1985;186:889-99.
23. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J. Triple immunological staining using colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.
24. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
25. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.
26. Van Dongen JJM, Comans-Bitter WM. Phenotypic and genotypic characteristics of the human prothymocyte. Immunol Res 1987;6:250-62.
27. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
28. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.
29. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
30. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.

31. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-28.
32. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T cell receptor delta genes in T cell acute lymphoblastic leukemias. Blood 1989;74:334-42.
33. Van Dongen JJM, Comans-Bitter WM, Friedrich W, Neijens HJ, Belohradsky BH, Kohn Th, Hagemeijer A, Borst J. Analysis of patients with DiGeorge anomaly provides evidence for extrathymic development of TcR-$\gamma\delta^+$ lymphocytes in man. Submitted for publication.
34. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA 1980;77:1588-92.
35. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
36. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-22.
37. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-5.
38. Landau NR, Schatz DG, Rosa M, Baltimore D. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol Cell Biol 1987;7:3237-43.
39. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
40. Link MP, Stewart SJ, Warnke RA, Levy R. Discordance between surface and cytoplasmic expression of the Leu-4 (T3) antigen in thymocytes and in blast cells from childhood T lymphoblastic malignancies. J Clin Invest 1985;76:248-53.
41. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
42. Lanier LL, Allison JP, Phillips JH. Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. J Immunol 1986;137:2501-7.
43. Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian W-T, Ip S, Wong L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. J Immunol 1989;142:57-66.
44. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB. Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 1989;169:1277-94.
45. Falini B, Flenghi L, Pileri S, Pelicci P, Fagioli M, Martelli MF, Moretta L, Ciccone E. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. J Immunol 1989;143:2480-8.
46. Campana D, Van Dongen JJM, Mehta A, Coustan-Smith E, Wolvers-Tettero ILM, Ganeshaguru K, Janossy G. The stages of T cell receptor protein expression in T cell acute lymphoblastic leukemia. Manuscript submitted.
47. Loiseau P, Guglielmi P, Le Paslier D, MacIntyre E, Gessain A, Bories J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;142:3305-11.
48. Gouttefangeas C, Bensussan A, Boumsell L. Study of the CD3-associated T-cell receptors reveals further differences between T-cell acute lymphoblastic lymphoma and leukemia. Blood 1990;75:931-4.
49. Hayday AC, Diamond DJ, Tanigawa G, Heilig JS, Folsom V, Saito H, Tonegawa S. Unusual organization and diversity of T-cell receptor α-chain genes. Nature 1985;316:828-32.
50. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.
51. Sims JE, Tunnacliffe A, Smith WJ, Rabbitts TH. Complexity of human T-cell antigen receptor β-chain constant- and variable-region genes. Nature 1984;312:541-5.

52. Toyonaga B, Yorshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β chain. Proc Natl Acad Sci USA 1985;82:8624-8.
53. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.
54. Lefranc M-P, Rabbitts TH. Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types. Nature 1985;316:464-6.
55. Lefranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
56. Lefranc M-P, Rabbitts TH. The human T-cell receptor γ (TRG) genes. Trends Biochem Sciences 1989;14:214-8
57. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-8.
58. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-7.
59. Satyanarayana K, Hata S, Devlin P, Grazia Roncarolo M, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-70.
60. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-101.
61. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. The human T-cell receptor delta chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
62. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
63. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
64. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-7.
65. Hata S, Satyanarayane K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-4.
66. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-8.
67. Krangel MS, Band H, Hata S, McLean J, Brenner MB. Structurally divergent human T cell receptor γ proteins encoded by distinct Cγ genes. Science 1987;237:64-7.
68. Lefranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-600.
69. Christmas SE. Most human $CD3^+WT31^-$ clones with T cell receptor Cγ1 rearrangements show strong non-MHC-restricted cytotoxic activity in contrast to those with Cγ2 rearrangements. Eur J Immunol 1989;19:741-6.
70. Grossi CE, Ciccone E, Migone N, Bottino C, Zarcone D, Mingari MC, Ferrini S, Tambussi G, Viale O, Casorati G, Millo R, Moretta L, Moretta A. Human T cells expressing the γ/δ T-cell receptor (TcR-1): Cγ1-and Cγ2-encoded forms of the receptor correlate with distinctive morphology, cytoskeletal organization, and growth characteristics. Proc Natl Acad Sci USA 1989;86:1619-23.
71. Greenberg JM, Quertermous T, Seidman JG, Kersey JH. Human T cell γ-chain gene rearrangements in acute lymphoid and nonlymphoid leukemia: comparison with the T cell receptor β-chain gene. J Immunol 1986;137:2043-9.
72. Davey MP, Bongiovanni KF, Kaulfersch W, Quertermous T, Seidman JG, Hershfield MS, Kurtzberg J, Haynes BF, Davis MM, Waldmann TA. Immunoglobulin and T-cell receptor gene rearrangement and expression in human lymphoid leukemia cells at different stages of maturation. Proc Natl Acad Sci USA 1986;83:8759-63.
73. Goorha R, Bunin N, Mirro J, Murphy SB, Cross AH, Behm FG, Quertermous T, Seidman J, Kitchingman GR. Provocative pattern of rearrangements of the genes for the γ and β chains of the T-cell receptor in human leukemias. Proc Natl Acad Sci USA 1987;84:4547-51.

74. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ. T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
75. Le Paslier D, Chen Z, Loiseau P, Cohen D, Sigaux F. T cell rearranging gene γ: diversity and mRNA expression in fresh cells from T cell acute lymphoblastic leukemia. Blood 1987;70:637-46.
76. Foroni L, Foldi J, Matutes E, Catovsky D, O'Connor NJ, Baer R, Forster A, Rabbitts TH, Luzzatto L. α, β and γ T-cell receptor genes: rearrangements correlate with haematological phenotype in T cell leukaemias. Br J Haematol 1987;67:307-18.
77. Miwa H, Konishi H, Kobayashi N, Kita K, Shirakawa S, Shimizu A, Honjo T, Hatanaka M. The T-cell receptor gene rearrangements in T-lineage tumors without OKT-3,4,6,8 markers. J Mol Cell Immunol 1987;3:37-42.
78. Tawa A, Benedict SH, Hara J, Hozumi N, Gelfand EW. Rearrangement of the T cell receptor γ-chain gene in childhood acute lymphoblastic leukemia. Blood 1987;70:1933-9.
79. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Comparison of T cell receptor α, β, and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J Clin Invest 1988;81:989-96.
80. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-82.
81. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor (TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-8.
82. Foroni L, Laffan M, Boehm T, Rabbitts TH, Catovsky D, Luzzatto L. Rearrangement of the T-cell receptor δ genes in human T-cell leukemias. Blood 1989;73:559-65.
83. Asou N, Hattori T, Matsuoka M, Kawano F, Takatsuki K. Rearrangements of T-cell antigen receptor δ chain gene in hematologic neoplasms. Blood 1989;74:2707-12.
84. Triebel F, Lefranc M-P, Hercend T. Further evidence for a sequentially ordered activation of T cell rearranging gamma genes during T lymphocyte differentiation. Eur J Immunol 1988;18:789-94.
85. Koning F, Maloy WL, Cohen D, Coligan JE. Independent association of T cell receptor β and γ chains with CD3 in the same cell. J Exp Med 1987;166:595-600.
86. Boehm TLJ, Werle A, Ganser A, Kornhuber B, Drahovsky D. T cell receptor γ chain variable gene rearrangements in acute lymphoblastic leukemias of T and B lineage. Eur J Immunol 1987;17:1593-7.
87. Rupp F, Frech G, Hengartner H, Zinkernagel RM, Joho R. No functional γ-chain transcripts detected in an alloreactive cytotoxic T-cell clone. Nature 1986;321:876-8.
88. Reilly EB, Kranz DM, Tonegawa S, Eisen HN. A functional γ gene formed from known γ-gene segments is not necessary for antigen-specific responses of murine cytotoxic T lymphocytes. Nature 1986;321:878-80.
89. Yoshikai Y, Toyonaga B, Koga Y, Kimura N, Griesser H, Mak TW. Repertoire of the human T cell gamma genes: high frequency of nonfunctional transcripts in thymus and mature T cells. Eur J Immunol 1987;17:119-26.
90. Chien Y, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature 1987;327:677-82.
91. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-7.
92. Lindsten T, Fowlkes BJ, Samelson LE, Davis MM, Chien Y-H. Transient rearrangements of the T cell antigen receptor α locus in early thymocytes. J Exp Med 1987;166:761-5.
93. Born W, Rathbun G, Tucker P, Marrack P, Kappler J. Synchronized rearrangement of T-cell γ and β chain genes in fetal thymocyte development. Science 1986;234:479-82.
94. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.
95. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
96. Snodgrass HR, Kisielow P, Kiefer M, Steinmetz M, Von Boehmer H. Ontogeny of the T-cell antigen receptor within the thymus. Nature 1985;313:592-5.
97. Born W, Yagüe J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor β-chain genes during T-cell development. Proc Natl Acad Sci USA 1985;82:2925-9.
98. Pardoll DM, Fowlkes BJ, Bluestone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential

expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
99. Bluestone JA, Pardoll D, Sharrow SO, Fowlkes BJ. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. Nature 1987;326:82-4.
100. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 1988;335:443-5.
101. Tonegawa S, Berns A, Bonneville M, Farr AG, Ishida I, Ito K, Itohara S, Janeway CA jr, Kanagawa O, Katsuiki M, Kubo R, Lafaille JJ, Mombaerts P, Murphy DB, Nakanishi N, Takagaki Y, Van Kaer L, Verbeek S. Diversity, development, ligands and probable functions of $\gamma\delta$ T cells. Progr Immunol 1989;7:243-57.
102. De Villartay J-P, Lewis D, Hockett R, Waldmann TA, Korsmeyer SJ, Cohen DI. Deletional rearrangement in the human T-cell receptor α-chain locus. Proc Natl Acad Sci USA 1987;84:8608-12.
103. De Villartay J-P, Hockett RD, Coran D, Korsmeyer SJ, Cohen DI. Deletion of the human T-cell receptor δ-gene by a site-specific recombination. Nature 1988;335:170-4.
104. Hockett RD, Nuñez G, Korsmeyer SJ. Evolutionary comparison of murine and human δ T-cell receptor deleting elements. New Biologist 1989;1:266-74.
105. Takeshita S, Toda M, Yamagishi H. Excision products of the T cell receptor gene support a progressive rearrangement model of the α/δ locus. EMBO J 1989;8:3261-70.
106. Begley CG, Aplan PD, Davey MP, De Villertay J-P, Cohen DI, Waldmann TA, Kirsch IR. Demonstration of δrec-pseudo Jα rearrangement with deletion of the δ locus in a human stem-cell leukemia. J Exp Med 1989;170:339-42.
107. Tonegawa S. Somatic generation of antibody diversity. Nature 1983;302:575-81.
108. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
109. Fujimoto S, Yamagishi H. Isolation of an excision product of T-cell receptor α-chain gene rearrangements. Nature 1987;327:242-3.
110. Okazaki K, Davis DD, Sakano H. T cell receptor β gene sequences in the circular DNA of thymocyte nuclei: direct evidence for intramolecular DNA deletion in V-D-J joining. Cell 1987;49:477-85.
111. Toda M, Fujimoto S, Iwasato T, Takeshita S, Tezuka K, Ohbayashi T, Yamagishi H. Structure of extra-chromosomal circular DNAs excised from T-cell antigen receptor alpha and delta-chain loci. J Mol Biol 1988;202:219-31.
112. Okazaki K, Sakano H. Thymocyte circular DNA excised from T cell receptor α-δ gene complex. EMBO J 1988;7:1669-74.
113. Winoto A, Baltimore D. Separate lineages of T cells expressing the $\alpha\beta$ and $\gamma\delta$ receptors. Nature 1989;338:430-2.
114. Winoto A, Baltimore D. A novel, inducible and T cell-specific enhancer located at the 3′ end of the T cell receptor α locus. EMBO J 1989;8:729-33.
115. Winoto A, Baltimore D. $\alpha\beta$ lineage-specific expression of the α T cell receptor gene by nearby silencers. Cell 1989;59:649-55.
116. Bories JC, Loiseau P, d'Auriol L, Gontier C, Bensussan A, Degos L, Sigaux F. Regulation of transcription of the human T cell antigen receptor δ chain gene: a T lineage-specific enhancer element is located in the Jδ3-Cδ intron. J Exp Med 1990;171:75-83.
117. Bonneville M, Ishida I, Mombaerts P, Katsuki M, Verbeek S, Berns A, Tonegawa S. Blockage of $\alpha\beta$ T-cell development by TcR $\gamma\delta$ transgenes. Nature 1989;342:931-4.
118. Ishida I, Verbeek S, Bonneville M, Berns A, Tonegawa S. T cell receptor $\gamma\delta$ transgenic mice suggest a role of a γ gene silencer in the generation of $\alpha\beta$ T cells. Proc Natl Acad Sci USA, in press.
119. Hochstenbach F, Brenner MB. T-cell receptor δ-chain can substitute for α to form a $\beta\delta$ heterodimer. Nature 1989;340:562-5.
120. Saito T, Hochstenbach F, Marusic-Galesic S, Kruisbeek AM, Brenner M, Germain RN. Surface expression of only $\gamma\delta$ and/or $\alpha\beta$ T cell receptor heterodimers by cells with four (α, β, γ, δ) functional receptor chains. J Exp Med 1988;168:1003-20.
121. De Waal Malefyt R, Alarcon B, Yssel H, Sancho J, Miyajima A, Terhorst CP, Spits H, De Vries JE. Introduction of T cell receptor (TcR)-α cDNA has differential effects on TcR-$\gamma\delta$/CD3 expression by peer and lyon-1 cells. J Immunol 1989;142:3634-42.
122. Strominger JL. Developmental biology of T cell receptors. Science 1989;244:943-50.

123. Yoshikai Y, Reis MD, Mak TW. Athymic mice express a high level of functional γ-chain but greatly reduced levels of α- and β-chain T cell receptor messages. Nature 1986;324:482-5.
124. Yoshikai Y, Matsuzaki G, Takeda Y, Ohga S, Kishihara K, Yuuki H, Nomoto K. Functional T cell recptor δ chain gene messages in athymic nude mice. Eur J Immunol 1988;18:1039-43.
125. Havran WL, Grell S, Duwe G, Kimura J, Wilson A, Kruisbeek AM, O'Brien RL, Born W, Tigelaar RE, Allison JP. Limited diversity of T-cell receptor γ-chain expression of murine Thy-1$^+$ dendritic epidermal cells revealed by Vγ3-specific monoclonal antibody. Proc Natl Acad Sci USA 1989;86:4185-9.
126. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-70.
127. Ito K, Bonneville M, Takagaki Y, Nakanishi N, Kanagawa O, Krecko EG, Tonegawa S. Different γδ T-cell receptors are expressed on thymocytes at different stages of development. Proc Natl Acad Sci USA 1989;86:631-5.
128. Itohara S, Nakanishi N, Kanagawa O, Kubo R, Tonegawa S. Monoclonal antibodies specific to native murine T-cell receptor γδ: analysis of γδ T cells during thymic ontogeny and in peripheral lymphoid organs. Proc Natl Acad Sci USA 1989;86:5094-8.
129. Asarnow DM, Goodman T, LeFrancois L, Allison JP. Distinct antigen receptor repertoires of two classes of murine epithelium-associated T cells. Nature 1989;341:60-2.
130. Nixon-Fulton JL, Kuziel WA, Santerse B, Bergstresser PR, Tucker PW, Tigelaar RE. Thy-1$^+$ epidermal cells in nude mice are distinct from their counterparts in thymus-bearing mice: a study of morphology, function, and T cell receptor expression. J Immunol 1988;141:1897-903.
131. Pardoll DM, Fowlkes BJ, Lew AM, Maloy WL, Weston MA, Bluestone JA, Schwartz RH, Coligan JE, Kruisbeek AM. Thymus-dependent and thymus-independent developmental pathways for peripheral T cell receptor-γδ-bearing lymphocytes. J Immunol 1988;140:4091-6.
132. Vroom TM, Bos JD, Borst J. Scientific correspondence. Nature 1989;341:114.
133. DiGeorge AM. Congenital absence of the thymus and its immunologic consequences: concurrence with congenital hypoparathyroidism. Birth Defects 1968;4(1):116-21.
134. Belohradsky BH. Thymusaplasie und -hypoplasie mit Hypoparathyreoidismus, Herz- und Gefaßmißbildungen (Di-George-Syndrom). Ergebnisse der Inneren Medizin und Kinderheilkunde 1985;54:35-105.
135. Lammer EJ, Opitz JM. The DiGeorge anomaly as a developmental field defect. Am J Med Genet 1986;2S:113-27.
136. Bastian J, Law S, Vogler L, Lawton A, Herrod H, Anderson S, Horowitz S,Hong R. Prediction of persistent immunodeficiency in the DiGeorge anomaly. J Pediatr 1989;115:391-6.
137. Steinmann GG, Klaus B, Müller-Hermelink H-K. The involution of the ageing human thymic epithelium is independent of puberty. Scand J Immunol 1985;22:563-75.
138. Steinmann GG. Changes in the human thymus during aging. Current Topics Pathol 1986;75:43-88.
139. Koning F, Knot M, Wassenaar F, Van den Elsen P. Phenotypical heterogeneity among human T cell receptor γ/δ-expressing clones derived from peripheral blood. Eur J Immunol 1989;19:2099-105.
140. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymfocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
141. Neudorf SML, Le Bien TW, Bollum F, Kersey JH. Double fluorochrome analysis of human bone marrow lymphoid cells: studies with terminal transferase and anti-T-cell monoclonal antibodies. Exp Hematol 1984;12:69-73.
142. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal rsidual disease in acute leukemia. Dordrecht: M Nijhoff Publishers, 1986:113-33.
143. Von Boehmer H, Kisielow P, Kishi H, Scott B, Borgulya P, Teh HS. The expression of CD4 and CD8 accessory molecules on mature T cells is not random but correlates with the specificity of the αβ receptor for antigen. Immunol Rev 1989;109:143-51.
144. Van Ewijk W, Kisielow P, Von Boehmer H. Immunohistology of T cell differentiation in the thymus of H-Y-specific T cell receptor α/β transgenic mice. Eur J Immunol 1990;20:129-37.

CHAPTER 4.2

HUMAN BONE MARROW CELLS POSITIVE FOR TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT), HLA-DR, AND A T CELL MARKER MAY REPRESENT PROTHYMOCYTES*

Jacques J.M. van Dongen[1], Herbert Hooijkaas[1], Marieke Comans-Bitter[1,2], Karel Hählen[3], Annelies de Klein[4], George E. van Zanen[3], Mars B. van 't Veer[5], Johan Abels[6] and Robbert Benner[1]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
2. Department of Pediatrics, Sophia Children's Hospital, Rotterdam;
3. Department of Pediatrics, Subdivision Hematology-Oncology;
4. Department of Cell Biology and Genetics;
5. Central Laboratory of the Blood Transfusion Service, Amsterdam;
6. Department of Hematology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands.

SUMMARY

Recent evidence suggests that prothymocytes, which occur in a low frequency in murine bone marrow (BM), are already committed to thymocyte differentiation and discrete from precursor B cells as well as pluripotent hematopoietic stem cells. Furthermore, it was suggested that, in rodents, prothymocytes are positive for the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) and a T cell surface antigen. The human prothymocyte has not been identified as yet. We analyzed human BM cells by double immunofluorescence staining for TdT and the T cell surface antigens CD1, CD2, CD5 and CD7. In the BM samples tested, neither $CD1^+/TdT^+$ nor $CD5^+/TdT^+$ cells were detected, but $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells were present in low frequencies. In childhood BM, the frequency was about two to five in 10,000, whereas in adult BM and regenerating BM, these cells were not always detectable, but if detected, their frequency was five- to 10-fold lower. In a triple staining, using fluorescein, rhodamine, and colloidal gold particles as labels, it appeared that all $CD7^+/TdT^+$ cells were also positive for HLA-DR. These $CD7^+/HLA\text{-}DR^+/TdT^+$ cells were also detectable in low frequencies in the thymus, and occasionally $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells were detected in the peripheral blood (PB), suggesting a migration from the BM to the thymus via the PB. The malignant counterpart of the $CD7^+/HLA\text{-}DR^+/TdT^+$ cell was detected in a patient with acute lymphoblastic leukemia with the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ phenotype and germline immunoglobulin heavy chain genes. We postulate that the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ cell represents a human prothymocyte.

* *Published in: J Immunol 1985;135:3144-50. Updated for CD codes.*

INTRODUCTION

Bone marrow (BM) and thymus are the breeding sites of B and T lymphocytes, respectively (1). Pre-B cells have been identified in the BM and fetal liver as large lymphoid cells with a small amount of cytoplasmic immunoglobulin (Ig) and without surface Ig (2). The precursors of the T lymphocytes, the so-called "prothymocytes", are also generated in the hematopoietic organs (3,4). Recent evidence suggests that prothymocytes, which occur in low frequencies in murine BM (0.015 to 0.03%) (5-7), are already committed to thymocyte differentiation and discrete from precursor B cells as well as pluripotent hematopoietic stem cells (8-10). Although the phenotype of the thymocyte is well described (1,4,11-13), the phenotype of the prothymocyte is not well known.

Several investigators suggest that rodent prothymocytes may be positive for the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) and a T cell surface antigen (10,14-19).

Jannossy and colleagues (20,21) have suggested that the human prothymocyte belongs to the pool of 0.3 to 10% TdT^+ cells in normal BM. However, cells positive for both a T cell marker and TdT have never been demonstrated in extrathymic locations such as BM, except in patients with T cell acute lymphoblastic leukemia (T-ALL) (13,20-23). Because thymus repopulation experiments in mouse and rat have demonstrated that the prothymocytes are extremely rare in the BM (5-7,17), we have standardized a method for identifying rare immature lymphoid cells by simultaneous detection of two or three markers (24,25). By using TdT, HLA-DR, and T cell surface antigens as markers, we analyzed BM, peripheral blood (PB), and thymus samples from children, BM samples from adults, and liver and BM samples from fetuses to determine whether immature T cells are detectable in these cell samples. In addition, the BM cells from a patient suffering from an ALL with a rare immunologic phenotype were studied.

MATERIALS AND METHODS

Cell samples

1. *BM and PB samples from children and adults.*

Thirty-nine BM samples were obtained from 34 children and five adults, and 10 PB samples were obtained from 10 children. The childhood BM samples were obtained from 12 children with precursor B-ALL (common ALL or pre-B-ALL) in complete remission under maintenance therapy, from four children with T-ALL under maintenance therapy (who were at least 6 mo in remission since the BM sampling), and from seven children with precursor B-ALL in complete remission, 6 wk after termination of maintenance therapy. In addition, BM samples were obtained from 11 children suffering from non-lymphoid malignancies with normal BM morphology, who did not receive any prior chemotherapy. The adult BM samples were obtained from five healthy volunteers (21 to 28 y old). The childhood PB samples were obtained from 10 children with precursor B-ALL in complete remission under maintenance therapy.

2. *Thymus samples.*

Four thymus samples were obtained from four children (<2 y old) undergoing cardiac surgery. These samples were minced with scissors in RPMI 1640 medium containing 10% fetal calf serum (FCS) and were flushed through a nylon gauze filter with 100-μm openings (Stokvis and Smits Textielmij, Haarlem, The Netherlands).

3. *Fetal liver and BM samples.*

Eight human fetuses of 14 to 19 wk gestational age were obtained by interruption of pregnancy on nonmedical indications. Fetal liver samples were minced with scissors in RPMI 1640 medium containing 10% FCS and were flushed through 100-μm nylon gauze. Fetal BM cells were sampled by flushing the long bones with a 10% FCS-RPMI solution; afterwards, the BM cells were flushed through 100-μm nylon gauze.

4. *BM sample from ALL patient at diagnosis.*

A BM sample was obtained from a 34-y-old man suffering from an acute leukemia. According to conventional morphology and cytochemistry, this leukemia appeared to be an ALL. The laboratory values at diagnosis revealed a hemoglobin of 5.4 mmol/l, platelets 90 x 10^9/l, and white blood cells 2.4 x 10^9/l, of which 71% were lymphoblasts. In the BM, all cells appeared to be lymphoblasts. Physical examination revealed discrete lymphadenopathy, no hepatosplenomegaly, and no mediastinal mass. The patient responded to daunorubicin-vincristine-cytarabine-prednisone therapy and remained in remission for 14 mo.

Immunologic characterization of cell samples

Mononuclear cells (MNC) from BM, PB, and fetal liver were isolated by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All washings were performed in phosphate-buffered saline (PBS) containing 1% bovine serum albumin (BSA), pH 7.8. For the immunologic characterization of the cell samples, we used an anti-TdT antiserum (26), an anti-HLA-DR monoclonal antibody (McAb) (27), the McAb WT1 (22,28) and 3A1 (29), both recognizing different epitopes of the same (41kDa) T cell antigen CD7 (30), the McAb OKT11 (31), and Leu-5 (32), both of which recognize the CD2 antigen (the sheep red blood cell receptor), the CD5 McAb Leu-1 (33), and the CD1 McAb 66IIIE5 (34). For additional characterization of the BM cells from the ALL patient, the following McAb were used: the T cell-specific McAb Leu-3a (35), Leu-2a (35), and Leu-4 (36), recognizing the CD4, CD8 and CD3 antigens respectively; the McAb VIL-A1 (37), which recognizes the CD10 antigen; the McAb BA-1 (38), BA-2 (39), B1 (40), and B2 (41), which recognize the B cell antigens CD24, CD9, CD20 and CD21 respectively; and the McAb VIM-D5 (42), My4 (43), My8 (43), My906 (44), and FMC17 (45), which recognize myelomonocytic antigens. (Further information about the various McAb used is given in Table 4) The McAb CD10 (VIL-A1) and CD24 (BA-1) were also used for additional characterization of the fetal liver and BM cells and the BM cells from the children with precursor B-ALL in complete remission, 6 wk after termination of maintenance therapy.

The cells were incubated with the relevant McAb, were washed, and were subsequently incubated with a fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse Ig antiserum.

Double immunofluorescence (IF) staining

Double IF stainings were performed for a T or B cell antigen and TdT. The CD1/TdT, CD2/TdT, CD5/TdT, and CD7/TdT double IF stainings were performed on the childhood BM and PB samples, the adult BM samples, and the fetal liver and BM samples. The HLA-DR/TdT, CD10/TdT, and CD24/TdT double IF stainings were performed on the fetal liver and BM samples and several infant BM samples, especially those from children with precursor B-ALL in complete remission, 6 wk after termination of maintenance therapy. The MNC were successively incubated with one of the optimally titrated anti-T cell or anti-B cell McAb and a tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig antiserum (Nordic Immunological Laboratories, Tilburg, The Netherlands) (24). Subsequently, cytocentrifuge preparations of these labeled cells were subjected to indirect IF staining with a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit Ig antiserum (Bethesda Research Laboratory, Gaithersburg, MD). The slides were mounted in glycerol/PBS (9:1) containing 1 mg phenylenediamine per milliliter to prevent fading of the fluorescence (46). For the detection of the CD7 antigen in the CD7/TdT double IF stainings, the McAb WT1 as well as 3A1 were used; the McAb OKT11 and Leu-5 were used for the detection of the CD2 antigen in the CD2/TdT double IF stainings. In recent studies, we demonstrated in dilution experiments that it is possible to detect one double-positive cell in 10,000 or even 100,000 MNC (24).

Triple immunologic staining

For the CD7/HLA-DR/TdT triple immunologic staining the MNC were successively incubated with the McAb 3A1 and a goat anti-mouse Ig antiserum, conjugated with colloidal gold particles of about 30 nm (Janssen Pharmaceutica, Beerse, Belgium). Subsequently, the cells were incubated with normal mouse serum (diluted 1 in 100) to block free antigen-binding sites of the goat anti-mouse Ig antiserum. Finally, the cells were incubated with a TRITC-conjugated anti-HLA-DR McAb (Becton Dickinson, Sunnyvale, CA) (25). The cells were centrifuged on slides and were subjected to the indirect IF staining with the rabbit anti-TdT antiserum and the FITC-conjugated goat anti-rabbit Ig antiserum (25).

Microscopes

Zeiss (Carl Zeiss, Oberkochen, FRG) and Leitz (Ernst Leitz Wetzlar, Wetzlar, FRG) microscopes were used for the evaluation of the immunofluorescence and the immunogold staining. The microscopes were equipped with HBO mercury lamps (Osram, Berlin, FRG), phase-contrast facilities, and filter combinations for the selective visualization of FITC and TRITC. The immunogold staining was evaluated by epi-illumination with polarized light (25).

Evaluation of cytocentrifuge preparations

In the double- as well as the triple-labeling experiments, at least two cytocentrifuge preparations were made, each containing at least 25,000 cells. The percentages of double- or triple-positive cells were calculated by determining the fraction of TdT^+ cells per MNC, followed by counting the TdT^+ cells which were also positive for the second marker in case of double staining or for the two additional markers in case of triple staining. At least 1000 TdT^+ cells were evaluated for each double staining. This condition could generally be met when counting two cytocentrifuge preparations. However, for the detection of $CD7^+/TdT^+$ cells, two CD7 (3A1)/TdT as well as two CD7 (WT1)/TdT preparations were used. For the detection of the CD2 antigen, two CD2 (OKT11)/TdT as well as two CD2 (Leu-5)/TdT preparations were counted.

DNA analysis

DNA was prepared (47) from human placenta, BM cells from the described ALL patient, BM cells from a patient with a precursor B-ALL, and PB cells from a patient with chronic myeloid leukemia (CML). The DNA was digested with the restriction enzymes *Hin*dIII, *Pvu*II, and *Bgl*II, was electrophoresed on a 0.7% agarose gel, and was blotted to nitrocellulose. *Hin*dIII- and *Eco*RI-*Hin*dIII-digested λ-DNA preparations were included as mol. mass markers. The filters were hybridized to a 3.5 kilobase (kb) *Eco*RI-*Hin*dIII fragment containing the human J segment of the μ-heavy chain clone H24 (48). Hybridization and washing procedures were carried out according to described methods (49). Recombinant plasmids were handled under PZEK 1 containments according to the National Institutes of Health Guidelines for Research Involving Recombinant DNA molecules.

RESULTS

Double IF staining for TdT and a T cell marker on BM cells from children and adults

In the BM from children under ALL maintenance therapy and in normal childhood BM, the percentages of TdT^+ cells were about 2 to 4%, whereas in the BM from children 6 wk after

TABLE 1. Analysis of human BM cells by double IF staining for a T cell marker and TdT.

BM samples	% Positive cells per MNC				
	% TdT^+ cells	% $CD2^+/TdT^+$ cells	% $CD7^+/TdT^+$ cells	% $CD1^+/TdT^+$ cells	% $CD5^+/TdT^+$ cells
BM from children with precursor B-ALL in complete remission under maintenance therapy (n=12)	2.2 ± 2.8[a] (0.2-10.5)	0.05 ± 0.08 (0-0.3)	0.04 ± 0.04 (0.005-0.12)	–[c]	–
BM from children with T-ALL under maintenance therapy and at least 6 mo in remission after the BM sampling (n=4)	2.4 ± 0.9 (1.4-3.6)	NT[b]	0.02 ± 0.02 (0.001-0.05)	–	–
BM from children with normal BM morphology (n=11)	3.9 ± 3.1 (1.1-10.8)	0.03 ± 0.03 (0.004-0.11)	0.02 ± 0.01 (0.001-0.04)	–	–
BM from children with precursor B-ALL in complete remission, 6 wk after termination of maintenance therapy (n=7)	6.5 ± 2.3 (4.1-10.2)	0.009 ± 0.006 (0.005-0.02)	0.007 ± 0.006 (0-0.015)	–	–
BM from healthy adult volunteers (21 to 28 y old) (n=5)	1.1 ± 0.4 (0.7-1.7)	0.004 ± 0.004 (0-0.009)	0.002 ± 0.002 (0-0.005)	–	–

a. Mean ± SD. The figures in parentheses represent the ranges of the percentages of positive cells.
b. NT, not tested.
c. No double-positive cells detectable in at least 50,000 cells analyzed.

termination of maintenance therapy (i.e. regenerating BM), the percentages of TdT^+ cells werehigher: about 6.5%. In normal adult BM, about 1% of the MNC were TdT^+. Cells positive for both the CD1 antigen and TdT or the CD5 antigen and TdT could not be detected in any of the BM samples tested. However, cells positive for both the CD2 antigen and TdT and the CD7 antigen and TdT were detected in almost all childhood and adult BM samples (Table 1). The frequency of the $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells in BM from children under ALL maintenance therapy and in normal childhood BM is about two to five in 10,000 (Table 1). However, in regenerating childhood BM and in normal adult BM, $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells were not always detectable, and if detected, their frequency was about five-to 10-fold lower than in normal childhood BM and childhood BM under ALL maintenance therapy (Table 1). Such low frequencies are around the detection limit of the double IF staining (24).

TABLE 2. Analysis of fetal liver and fetal BM cells by single and double IF staining.

Fetal cell samples	% TdT^+ cells per MNC	% HLA-DR^+ cells per TdT^+ cells	% $CD10^+$ cells per TdT^+ cells	% $CD24^+$ cells per TdT^+ cells	% $CD10^+$ cells per MNC	% $CD24^+$ cells per MNC
Fetal liver	4.1 ± 1.6[a] (2.5-6.6)	97 ± 2.5 (92.5-99.5)	94.4 ± 2.5 (90-97.5)	99.9 ± 0.2 (99.5-100)	13.1 ± 7.9 (5.2-28.5)	18.4 ± 7.4 (9.0-32.5)
Fetal BM	13.9 ± 6.2 (3.4-24)	97.6 ± 2.2 (94.5-99.5)	91.7 ± 4.6 (85.5-97.5)	99.1 ± 1.2 (97.5-100)	27.1 ± 14.9 (6.6-46.2)	31.9 ± 13.5 (14.5-47.5)

a. Mean ± SD. The figures in parentheses represent the ranges of the percentages of positive cells.

Double IF staining for TdT and a T cell marker on PB cells from children

In the 10 PB samples from children with precursor B-ALL under maintenance therapy, the percentages of TdT^+ cells ranged from 0.01 to 0.2%. Only occasionally could $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells be detected. $CD1^+/TdT^+$ and $CD5^+/TdT^+$ cells were never detected in PB samples tested.

Double IF staining for TdT and a T cell marker, TdT and a B cell marker, or TdT and HLA-DR on fetal liver and fetal BM cells

In the fetal liver, about 4% of the MNC was TdT^+, whereas in the fetal BM, about 14% TdT^+ MNC were found. Neither in the fetal liver cell samples nor in the fetal BM cell samples could T cell $marker^+/TdT^+$ cells be detected in at least 1000 TdT^+ cells. Double staining for TdT and HLA-DR, CD10, or CD24 revealed that almost all (>92%) TdT^+ cells were also positive for the HLA-DR and CD24 antigen, whereas more than 85% of the TdT^+ cells were also positive for the CD10 antigen (Table 2). Furthermore, many TdT^- cells were also positive for the CD10 and CD24 antigen (Table 2). These data indicate that almost all TdT^+ cells are precursor B cells.

Double IF staining for TdT and the CD10 antigen on infant BM cells

In BM samples from children 6 wk after termination of the ALL maintenance therapy, about 6.5% of the MNC were positive for TdT, and more than 85% of these TdT^+ cells were also positive for the CD10 antigen. Many TdT^- cells were also positive for the CD10 antigen (up to 40% of the MNC). Furthermore, a high percentage of $CD24^+$ cells (>30%) was detected.

In BM samples from children under maintenance therapy and normal infant BM samples, the percentages of TdT^+ cells were lower (2 to 4%), and a smaller part of the TdT^+ cells was also positive for the CD10 antigen. Almost all $CD10^+$ cells were also positive for TdT, and only

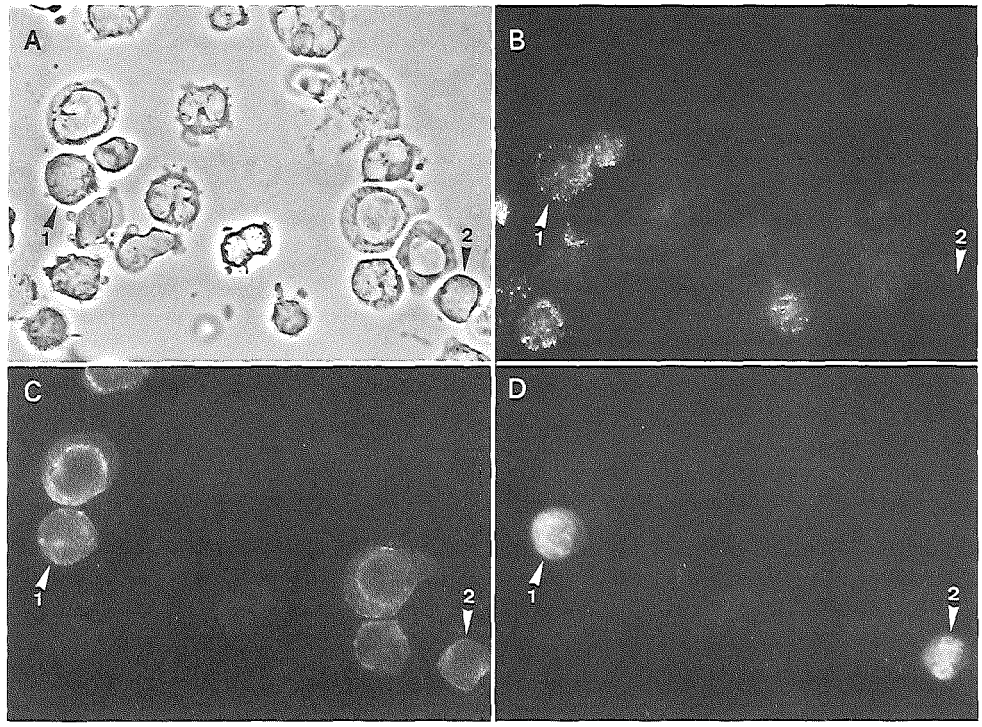


Figure 1. Analysis of mononuclear BM cells from a child with precursor B-ALL in complete remission under maintenance therapy, by a triple staining for CD7, HLA-DR, and TdT, using FITC, TRITC, and colloidal gold particles as labels. **A**: Phase-contrast picture of the BM cells; **B**: $CD7^+$ cells (labeled with colloidal gold particles); **C**: $HLA\text{-}DR^+$ cells (TRITC-labeled); **D**: TdT^+ cells (FITC-labeled). **A, B, C,** and **D** represent the same field. One cell (1) is positive for TdT, HLA-DR, and CD7 and another cell (2) is positive for TdT and HLA-DR but negative for CD7. The latter might be a precursor B cell (c.f. references 20 and 51), and the former might be a prothymocyte.

a small percentage of $CD24^+$ cells (<10%) was detected in these BM samples.

These data indicate that almost all TdT^+ cells in regenerating BM are precursor B cells, whereas in normal infant BM and BM from children under maintenance therapy, a smaller part of the TdT^+ cells are precursor B cells.

Triple immunologic staining for the CD7 antigen, HLA-DR, and TdT

Because most TdT^+ cells in BM are also positive for HLA-DR (20), we investigated whether the $CD7^+/TdT^+$ cells are also positive for HLA-DR. In the four BM samples from children under ALL maintenance therapy and in the two normal adult BM samples tested, all $CD7^+/TdT^+$ cells appeared to be also positive for HLA-DR (Figure 1; Table 3). In addition, we investigated whether $CD7^+/HLA\text{-}DR^+/TdT^+$ cells occur in the thymus as well. The triple-staining method revealed that large blasts with the $CD7^+/HLA\text{-}DR^+/TdT^+$ phenotype were detectable in a

TABLE 3. Analysis of BM and thymus samples by triple immunologic staining for CD7, HLA-DR, and TdT.

Triple immunologic staining	BM from children with precursor B-ALL in complete remission under maintenance therapy (n=4)	BM from healthy adult volunteers (n=2)	Thymus from children undergoing cardiac surgery (n=4)
% $CD7^+/HLA\text{-}DR^+/TdT^+$ cells per MNC	0.05 ± 0.03[a] (0.01-0.07)	0.003 ± 0.003 (0.001-0.006)	0.02 ± 0.01 (0.009-0.03)

a. Mean ± SD. The figures in parentheses represent the ranges of the percentages of positive cells.

frequency of 0.009 to 0.03% (Table 3). This special population represented 0.1 to 0.4% of the large $CD7^+/TdT^+$ blasts.

Immunologic marker analysis and Ig heavy chain gene analysis of the BM cells from the ALL patient at diagnosis

The immunologic marker analysis of the BM cells from the ALL patient revealed that most cells were positive for TdT, HLA-DR, the CD2 antigen, and the CD7 antigen, but negative for all other T cell markers and negative for all B cell and myelomonocytic markers tested (Table 4).

Southern blot analysis of the Ig heavy chain genes revealed that the BM cells from the ALL patient had germline Ig heavy chain genes. The DNA from human placenta and from the PB from a CML patient had germline Ig heavy chain genes, whereas the DNA from the BM cells from a common ALL patient had rearranged Ig heavy chain genes (Figure 2).

DISCUSSION

The phenotype of the human prothymocyte is not known, although it was suggested that this cell may be positive for TdT (21). Until now, however, cells positive for both a T cell marker and TdT have never been demonstrated in extrathymic locations such as BM (13,20-23).

By use of double IF stainings, we could detect $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells in very low frequencies in almost all childhood and adult BM samples tested. The frequency of the $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells in normal childhood BM and BM from children under ALL maintenance therapy was about two to five in 10,000 MNC. In adult BM and regenerating childhood BM, these cells were not always detectable, but if detected, their frequency was five- to 10-fold lower. Occasionally, the $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells could be detected in childhood PB. $CD1^+/TdT^+$ or $CD5^+/TdT^+$ cells were never detected in any of the BM and PB samples tested. No T cell $marker^+/TdT^+$ cells could be detected in the liver and BM samples from fetuses of 14 to 19 wk gestational age. Almost all TdT^+ cells in regenerating childhood BM and fetal liver and BM appeared to be precursor B cells.

TABLE 4. Immunologic marker analyse of the BM cells from the ALL patient.

Cluster of differentiation[a]	Antibodies	% Positive cells	Antigen recognized	References
–	anti-TdT[b]	92	TdT	26
–	anti-HLA-DR[c]	74	HLA-DR, nonpolymorphic antigen	27
CD7	WT1[d]	78	Tp41 antigen	22,28,30
CD7	3A1[e]	92	Tp41 antigen	29,30
CD2	OKT11[f]	94	T11 antigen	31
CD2	Leu-5[c]	90	T11 antigen	32
CD5	Leu-1[c]	2	T1 antigen	33
CD1	66IIIE5[g]	0	T6 antigen	34
CD4	Leu-3a[c]	0.5	T4 antigen	35
CD8	Leu-2a[c]	1.5	T8 antigen	35
CD3	Leu-4[c]	1.5	T3 antigen	36
CD10	VIL-A1[h]	0	common ALL antigen	37
CD24	BA-1[i]	0.5	B cell antigen	38
CD9	BA-2[i]	0	B cell antigen	39
CD20	B1[j]	0	B cell antigen	40
CD21	B2[j]	0	B cell antigen	41
CD15	VIM-D5[h]	1	myeloid antigen	42
CD14	My4[k]	2	monocytic antigen	43,44
CD11	My8[k]	0	myelomonocytic antigen	43,44
CD33	My906[k]	0.5	myelomonocytic antigen	44
CD14	FMC17[l]	1.5	monocytic antigen	45

a. Cluster of differentiation, as established during the Leucocyte Typing Conferences (Paris, 1982; Boston, 1984: Oxford, 1986).
b. Bethesda Research Laboratory, Gaithersburg, MD.
c. Becton Dickinson, Sunnyvale, CA.
d. Dr. W. Tax, Nijmegen, The Netherlands.
e. American Type Culture Collection, Rockville, MD.
f. Ortho Diagnostic Systems, Raritan, NJ.
g. Dr. J.M. van de Rijn, Amsterdam, The Netherlands.
h. Dr. W. Knapp, Vienna, Austria.
i. Hybritech, San Diego, CA.
j. Coulter Clone, Hialeah, FL.
k. Dr. J.D. Griffin, Boston, MA.
l. Sera Laboratories, Crawly Down, UK.

The CD2 and CD7 antigens are the most consisted T cell markers, and therefore $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells may represent precursor T cells. Because human hematopoietic progenitors are positive for HLA-DR (50), we investigated whether the $CD7^+$ cells in the BM belong to the $HLA\text{-}DR^+/TdT^+$ or the smaller $HLA\text{-}DR^-/TdT^+$ cell population (21). A triple staining for CD7, HLA-DR, and TdT was performed, using fluorescein, rhodamine, and colloidal gold particles as labels (25). All $CD7^+/TdT^+$ cells in the BM proved to be positive for HLA-DR as well. To investigate whether similar $CD7^+/HLA\text{-}DR^+/TdT^+$ cells also occur in the normal thymus, we analyzed thymus samples from four children. Previous studies have already shown that large $CD7^+$ blasts, which constitute 5% of all thymocytes strongly express TdT in the infant thymus (21), but it was still unknown whether a subset of these cells also express HLA-DR. Our triple-staining method revealed that large blasts of the $CD7^+/HLA\text{-}DR^+/TdT^+$ cell phenotype were found in a frequency of 0.009 to 0.03% (Table 3).

Finally, we studied leukemic BM cells from a 34-y-old man which proved to have the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ phenotype. This patient had an ALL according to

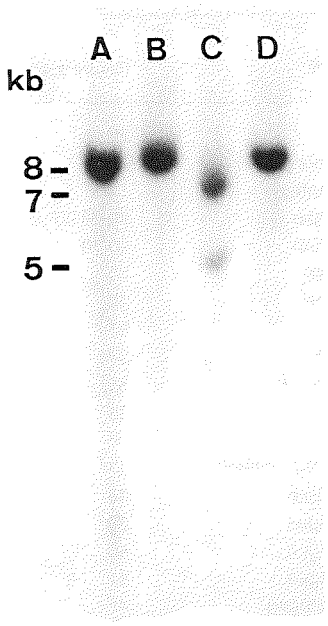


Figure 2. Southern blot analysis of Ig heavy chain genes. *Hin*dIII-digested DNA was prepared from human placenta (lane A), from BM cells from the described patient with the ALL of the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ phenotype (lane B), from BM cells from a patient with precursor B-ALL (lane C), and from PB cells from a patient with CML (lane D). The sizes of the germline bands (8 kb; lanes A,B, and D) and rearranged bands (7 and 5 kb; lane C) are indicated.

conventional morphology and cytochemistry, which responded to ALL therapy. DNA analysis revealed that the leukemic cells of this patient had germline Ig heavy chain genes. To our knowledge, no other $HLA\text{-}DR^+$ ALL without Ig gene rearrangements has been reported. This corresponds to the absence of Ig gene rearrangements in T cell leukemias (51,52), and indicates that the described $HLA\text{-}DR^+$ ALL is not a precursor B-ALL. These data suggest that the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ leukemia originated from a precursor T cell, probably a prothymocyte. T cell $marker^+/HLA\text{-}DR^+/TdT^+$ leukemias are rare (53), because only three other cases have been reported without, however, describing the Ig genes (24,54,55).

It must be mentioned that some papers describe $CD2^-$ precursor T cell leukemias (22,53). More mature $CD5^+$ T cell leukemias that are negative for the CD2 antigen have also been described (54). Furthermore, several sheep red blood cell receptor-negative T cell leukemias proved to be positive for the CD2 antigen, as detected by the McAb OKT11 (54). These discrepancies might be due to the weak expression of the CD2 antigen on immature T cell leukemias.

The phenotype of the putative prothymocytic leukemia corresponds to that of the normal $CD7^+/HLA\text{-}DR^+/TdT^+$ cell in human BM and thymus, which cells consequently may represent a normal prothymocyte. The frequencies of the $CD7^+/HLA\text{-}DR^+/TdT^+$ and $CD2^+/TdT^+$ cells in normal childhood infant BM and BM from children under maintenance therapy (Tables 1 and 3) are consistent with the frequency of the prothymocyte in murine BM (5- to 6-wk-old mice), as found by Kadish and Basch (0.015 to 0.02%) (5), Lepault and Weissman (<0.1%) (6), and Boersma et al. (0.03%)(7). Furthermore, it has been described that the frequency of the murine

prothymocyte is strongly reduced in regenerating BM (5,7). Boersma et al. (7) demonstrated that after 8 wk of BM regeneration, the frequency of the murine prothymocyte in the BM was about 16% of the normal frequency. This corresponds to five- to 10-fold lower frequencies of the $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells in the regenerating BM of children 6 wk after termination of maintenance therapy, as compared to normal childhood BM or BM from children under maintenance therapy. Boersma (56) also demonstrated that the frequency of the prothymocyte in the murine fetal liver is about 10% of the frequency of the prothymocyte in the BM of 6-wk-old mice. This low frequency might explain that no T cell $marker^+/TdT^+$ cells could be detected in human fetal liver. The latter finding corresponds to the data of Bodger et al. (57) that TdT^+ cells are detectable only in thymuses from human fetuses older than 20 wk gestational age.

On the basis of these data, we postulate that the human prothymocyte has the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ phenotype. Furthermore, the presence of $CD2^+/TdT^+/HLA\text{-}DR^+/TdT^+$ cells in the BM and the thymus, and the occasional detection of $CD2^+/TdT^+$ and $CD7^+/TdT^+$ cells in the PB, is consistent with the hypothesis that prothymocytes migrate from the BM via the PB to the thymus.

The difference between our human data and the rodent data is that no functional data are available about human precursor T cells, whereas all knowledge concerning the rodent prothymocyte is based on functional tests, but without further establishment of the precise phenotype. Nevertheless, our observations are very suggestive, especially due to the fact that a malignant counterpart of the $CD2^+/CD7^+/HLA\text{-}DR^+/TdT^+/CD1^-/CD5^-$ prothymocyte exists.

The establishment of the phenotype of the human prothymocyte may provide more insight into human T lymphocyte differentiation. The triple-staining method will be useful for studying the differentiation of T cells and other cell lineages *in vitro*, e.g. in cell clusters derived from T cell colony-forming cells. Also, the migration pattern of the prothymocyte in rodents can be studied by using the two- or threefold staining technique. Furthermore, it should be possible to purify murine prothymocytes by fluorescence-activated cell sorting with the use of markers analogous to the ones applied in the present study, and to conduct thymus repopulation experiments (5-7) with these cells.

ACKNOWLEDGMENTS. We gratefully acknowledge Dr. A. Hagemeijer and Dr. G. Grosveld for their support; Mrs. K. Benne, E.G.M. Cristen, I. Dekker, A.A. van der Linde-Preesman, and I.L.M. Wolvers-Tettero for their skillful technical assistance; Mr. T.M. van Os for his dedicated photographic assistance; Prof. Dr. E. Bos, Mrs. A.J. Meijer-Heijnsdijk, Dr. D.A.F. van Lith, Dr. K.J. van Schie, and Dr. J.J. Michiels for providing the cell samples; Dr. J. de Mey from Janssen Pharmaceutica for his advice; Mr. C. Enkelaar from Zeiss Nederland for his technical support; and Mrs. M. Stout for typing this manuscript.

This work was supported by the Netherlands Cancer Foundation.

REFERENCES

1. Goldschneider I. Early stages of lymphocyte development. Curr Top Dev Biol 1980;14:33-57.
2. Raff MC, Megson M, Owen JJT, Cooper MD. Early production of intracellular IgM by B-lymphocyte precursors in mouse. Nature 1976;259:224-6.
3. Ford CE, Micklem HS. The thymus and lymph-nodes in radiation chimaeras. Lancet 1963;i:359-62.
4. Scollay R. Intrathymic events in the differentiation of T lymphocytes: a continuing enigma. Immunol Today

1983;4:282-6.
5. Kadish JL, Basch RS. Hematopoietic thymocyte precursors. I. Assay and kinetics of the appearance of progeny. J Exp Med 1976;143:1082-99.
6. Lepault F, Weissman IL. An in vivo assay for thymus-homing bone marrow cells. Nature 1981;293:151-4.
7. Boersma W, Betel I, Daculsi R, Van der Westen G. Post-irradiation thymocyte regeneration after bone marrow transplantation. I. Regeneration and quantification of thymocyte progenitor cells in the bone marrow. Cell Tissue Kinet 1981;14:179-96.
8. Komuro K, Boyse EA. In-vitro demonstration of thymic hormone in the mouse by conversion of precursor cells into lymphocytes. Lancet 1973;i:740-3.
9. Basch RS, Goldstein G. Induction of T-cell differentiation in vitro by thymin, a purified polypeptide hormone of the thymus. Proc Natl Acad Sci USA 1974;71:1474-8.
10. Basch RS, Kadish JL, Goldstein G. Hematopoietic thymocyte precursors. IV. Enrichment of the precursors and evidence for heterogeneity. J Exp Med 1978;146:1843-8.
11. Tidman N, Janossy G, Bodger M, Granger S, Kung PC, Goldstein G. Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. Clin Exp Immunol 1981;45:457-67.
12. Barton R, Goldschneider I, Bollum FJ. The distribution of terminal deoxynucleotidyl transferase (TdT) among subsets of thymocytes in the rat. J Immunol 1976;116:462-8.
13. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
14. Silverstone AE, Cantor H, Goldstein G, Baltimore D. Terminal deoxynucleotidyl transferase is found in prothymocytes. J Exp Med 1976;144:543-8.
15. Pazmino NH, Ihle JN, Goldstein AL. Induction in vivo and in vitro of terminal deoxynucleotidyl transferase by thymosin in bone marrow cells from athymic mice. J Exp Med 1978;147:708-18.
16. Goldschneider I, Ahmed A, Bollum FJ, Goldstein AL. Induction of terminal deoxynucleotidyl transferase and Lyt antigens with thymosin: identification of multiple subsets of prothymocytes in mouse bone marrow and spleen. Proc Natl Acad Sci USA 1981;78:2469-73.
17. Greiner DL, Goldschneider I, Barton RW. Identification of thymocyte progenitors in hemopoietic tissues of the rat. II. Enrichment of functional prothymocytes on the fluorescence-activated cell sorter. J Exp Med 1982;156:1448-60.
18. Barton RW. The binding of Maclura pomifera lectin to cells of the T-lymphocyte lineage in the rat. Cell Immunol 1982;67:101-11.
19. Hayashi J, Medlock ES, Goldschneider I. A selective culture system for generating terminal deoxynucleotidyl transferase-positive (TdT^+) lymphoid precursor cells in vitro. I. Description of the culture system. J Exp Med 1984;160:1622-39.
20. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combination. Blood 1980;56:430-41.
21. Janossy G, Prentice HG. T cell subpopulations, monoclonal antibodies and their therapeutic applications. In: Janossy G, ed. Clinics in Haematology. London: Saunders Company, 1982;11:631-60.
22. Vodinelich L, Tax W, Bai Y, Pegram S, Capel P, Greaves MF. A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 1983;62:1108-13.
23. Neudorf SML, Le Bien TW, Bollum F, Kersey JH. Double fluorochrome analysis of human bone marrow lymphoid cells: studies with terminal transferase and anti-T-cell monoclonal antibodies. Exp Hematol 1984;12:69-73.
24. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: Martinus Nijhoff Publishers, 1984:67-81.
25. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J. Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985:80:1-6.
26. Goldschneider I, Gregoire KE, Barton RW, Bollum FJ. Demonstration of terminal deoxynucleotidyl transferase

in thymocytes by immunofluorescence. Proc Natl Acad Sci USA 1977;74:734-8.
27. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:293-9.
28. Tax WJM, Tidman N, Janossy G, Trejdosiewicz L, Willems R, Leeuwenberg J, De Witte TJM, Capel PJA, Koene RAP. Monoclonal antibody (WT1) directed against a T cell surface glycoprotein: characteristics and immunosuppressive activity. Clin Exp Immunol 1984;55:427-36.
29. Haynes BF, Mann DL, Hemler ME, Schroer JA, Shelhamer JH, Eisenbarth GS, Strominger JL, Thomas CA, Mostowski HS, Fauci AS. Characterization of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans. Proc Natl Acad Sci USA 1980;77:2914-8.
30. Hansen JA, Martin PJ, Beatty PC, Clark EA, Ledbetter JA. Human T lymphocyte cell surface molecules defined by the workshop monoclonal antibodies ('T cell protocol'). In: Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte Typing. Berlin: Springer Verlag, 1984:195-212.
31. Verbi W, Greaves MF, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G. Monoclonal antibodies OKT11 and OKT11A have pan-T reactivity and block sheep erythrocyte "receptors". Eur J Immunol 1982;12:81-6.
32. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J Immunol 1981;126:2117-22.
33. Martin PJ, Hansen JA, Siadak AW, Nowinski RC. Monoclonal antibodies recognizing normal human T lymphocytes and malignant human B lymphocytes: a comparative study. J Immunol 1981;127:1920-3.
34. Lerch PG, Van de Rijn M, Schrier P, Terhorst C. Biochemical comparison of the T6 antigen and HLA-A, B antigens. Hum Immunol 1983;6:13-30.
35. Engleman EG, Benike CJ, Glickman E, Evans RL. Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man. J Exp Med 1981;153:193-8.
36. Borst J, Prendiville MA, Terhorst C. Complexity of the human T lymphocyte-specific cell surface T3. J Immunol 1982;128:1560-5.
37. Knapp W, Majdic O, Bettelheim P, Liszka K. VIL-A1, a monoclonal antibody reactive with common acute lymphatic leukemia cells. Leuk Res 1982;6:137-47.
38. Abramson CS, Kersey JH, LeBien TW. A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. J Immunol 1981;126:83-8.
39. Kersey JH, LeBien TW, Abramson CS, Newman R, Sutherland R, Greaves M. p24: a human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. J Exp Med 1981;153:726-31.
40. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. J Immunol 1980;125:1678-85.
41. Nadler LM, Stashenko P, Hardy R, Van Agthoven A, Terhorst C, Schlossman SF. Characterization of a human B cell-specific antigen (B2) distinct from B1. J Immunol 1981;126:1941-7.
42. Majdic O, Liszka K, Lutz D, Knapp W. Myeloid differentiation antigen defined by a monoclonal antibody. Blood 1981;58:1127-33.
43. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557-63.
44. Griffin JD, Schlossman SF. Expression of myeloid differentiation antigens in acute myeloblastic leukemia. In: Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Berlin: Springer-Verlag, 1984:404-10.
45. Hancock WW, Zola H, Atkins RC. Antigenic heterogeneity of human mononuclear phagocytes: immunohistologic analysis using monoclonal antibodies. Blood 1983;62:1271-9.
46. Johnsen GD, De C Nogueira Araujo GM. A simple method of reducing the fading of immunofluorescence during microscopy. J Immunol Methods 1981;43:349-50.
47. Jeffreys AJ, Flavell RA. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell 1977;12:429-39.
48. Takahashi N, Nakai S, Honjo T. Cloning of human immunoglobulin μ gene and comparison with mouse μ

gene. Nucleic Acids Res 1980;8:5983-91.
49. Bernards R, Flavell RA. Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglobin (HPFH). Nucleic Acids Res 1980;8:1521-34.
50. Falkenburg JHF, Jansen J, Van der Vaart-Duinkerken N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ. Polymorphic and monomorphic HLA-DR determinants on human hematopoietic progenitor cells. Blood 1984;63:1125-32.
51. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
52. Ford AM, Molgaard HV, Greaves MF, Gould HJ. Immunoglobulin gene organisation and expression in haemopoietic stem cell leukaemia. EMBO J 1983;2:997-1001.
53. Greaves MF, Rao J, Hariri G, Verbi W, Catovsky D, Kung P, Goldstein G. Phenotypic heterogeneity and cellular origins of T cell malignancies. Leuk Res 1981;5:281-99.
54. Chen P-M, Chiang H, Chou C-K, Hwang T-S, Chiang BN, Greaves MF. Immunological classification of T-cell acute lymphoblastic leukaemia using monoclonal antibodies. Leuk Res 1983;7:339-48.
55. Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY. Immunoenzymatic staining of haematological samples with monoclonal antibodies. Br J Haematol 1983;55:395-410.
56. Boersma WJA. Prothymocytes in mouse fetal liver. Thymus 1983;5:419-28.
57. Bodger MP, Janossy G, Bollum FJ, Burford GD, Hoffbrand AV. The ontogeny of terminal deoxynucleotidyl transferase positive cells in the human fetus. Blood 1983;61:1125-31.

CHAPTER 4.3

T CELL RECEPTOR - CD3 COMPLEX DURING EARLY T CELL DIFFERENTIATION: ANALYSIS OF IMMATURE T CELL ACUTE LYMPHOBLASTIC LEUKEMIAS (T-ALL) AT DNA, RNA AND CELL MEMBRANE LEVEL*

Jacques J.M. van Dongen[1], Thomas Quertermous[2], Claus R. Bartram[3], Daniel P. Gold[4], Ingrid L.M. Wolvers-Tettero[1], W. Marieke Comans-Bitter[1,5], Herbert Hooijkaas[1], Henk J. Adriaansen[1], Annelies de Klein[6], Anand Raghavachar[7], Arnold Ganser[8], Allan D. Duby[2], John G.Seidman[2], Peter van den Elsen[4] and Cox Terhorst[4]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands;
2. Department of Genetics of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA;
3. Department of Pediatrics II, University of Ulm, Ulm, FRG; 4. Laboratory of Molecular Immunology of the Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA;
5. Department of Pediatrics, Sophia Children's Hospital, Rotterdam, The Netherlands;
6. Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands;
7. Department of Transfusion Medicine, University of Ulm, Ulm, FRG;
8. Department of Hematology, University of Frankfurt, Frankfurt, FRG.

SUMMARY

T cell acute lymphoblastic leukemias (T-ALL) can be regarded as the malignant counterparts of cells in various T cell differentiation stages. To study the expression of the human T cell receptor (TcR)-CD3 complex during the early stages of T cell differentiation, we have analyzed 22 T-ALL at the cell membrane level and the DNA level and 12 of them at the RNA level. According to their immunologic phenotype, the T-ALL could be divided into three main groups: 10 immature T-ALL ($CD1^-/CD3^-$), 7 common thymocytic T-ALL ($CD1^+/CD3^{-\text{ or }+}$) and five mature T-ALL ($CD1^-/CD3^+$). Among the ten immature T-ALL three appeared to express the immunologic phenotype of the putative prothymocyte ($TdT^+/HLA\text{-}DR^+/CD2^+/CD7^+/CD1^-/CD3^-/CD5^-$), whereas the other seven T-ALL appeared to be immature thymocytic ($TdT^+/HLA\text{-}DR^-/CD7^+/CD2^+/CD5^+/CD1^-/CD3^-$).

Transcripts of the CD3-δ and CD3-ϵ genes were present in all $CD3^-$ and $CD3^+$ T-ALL tested, including prothymocytic T-ALL. However, the prothymocytic T-ALL had germline TcR-β genes and were not rearranged to the characterized TcR-γ joining regions. The presence of CD3 transcripts and absence of TcR gene rearrangements in prothymocytic T-ALL supports

* *Published in: J Immunol 1987;138:1260-9.*

their immature T cell character. Two immature thymocytic T-ALL also had germline TcR-γ genes and one of them had germline TcR-β genes. In all other T-ALL the TcR-γ and TcR-β genes were rearranged. The presumptive functional 1.3-kb TcR-β transcripts were detected in the majority of T-ALL with rearranged TcR-β genes. Distinct levels of TcR-γ transcripts appeared to be present only in some thymocytic T-ALL, i.e. some immature thymocytic T-ALL and common thymocytic T-ALL. TcR-α mRNA could only be detected in $CD3^+$ mature T-ALL, but was absent in all $CD3^+$ common thymocytic T-ALL tested.

Our data indicate that CD3 gene transcription is one of the earliest events during T cell differentiation and already occurs in prothymocytes. The TcR-γ and TcR-β genes rearrange early during thymocytic differentiation and can subsequently be transcribed. High levels of TcR-γ gene transcription may only occur in a part of the T cells during thymic differentiation, while TcR-β gene transcription continues during further differentiation. TcR-α gene transcription may be the final step in the production of the complete set of TcR and CD3 proteins, resulting in the expression of the TcR-αβ-CD3 complex at the cell surface of mature T cells. Our data point to a predetermined order of rearrangement and expression of the TcR and CD3 genes during early stages of human T cell differentiation.

In the $CD3^+$ common thymocytic T-ALL tested no TcR-α transcripts could be detected, while TcR-β transcripts were present in all three T-ALL and TcR-γ transcripts in two of them. Since the expression of CD3 on the cell surface seems to be dependent on the presence of the TcR chains, these data suggest that in these T-ALL the TcR may be formed by a TcR-γ and a TcR-β chain, or that an alternate or aberrant TcR was present in these T-ALL.

INTRODUCTION

Recently much information has become available about the mouse and human antigen-specific T cell receptor (TcR)(1-3). The TcR, expressed on the cell surface of most mature T lymphocytes, consists of two glycoprotein chains, the TcR-α-chain (49 kDa) and TcR-β-chain (43 kDa)(4,5). Analogous to immunoglobulins, the diversity of the TcR-α- and -β-chains is based on the use of different variable, diversity and joining regions, which become linked by gene rearrangement (6-12). It is postulated that the rearrangement of the TcR-α and -β genes takes place during the differentiation/maturation process in the thymus (13-15). Similar to the Ig gene rearrangement during B cell differentiation (16), ordered rearrangement and transcription of the TcR genes seems to exist, i.e. the TcR-β genes rearrange and are transcribed prior to the TcR-α genes (15,17-20).

In addition to the TcR-α and -β genes, a third rearranging T cell receptor like gene, TcR-γ, has been described (21-28). Data from studies in the mouse indicate that TcR-γ gene transcription takes place early in the T cell differentiation, most probably prior to TcR-β gene transcription (17,18). In mice and humans, the TcR-γ gene rearrangement and transcription seems to be T cell specific (17,18,24-26). Although, the encoded protein has recently been identified, the precise function of the TcR-γ-chain has still to be elucidated (29). The appearance of TcR-γ-chain transcripts early in thymic development has led to the suggestion that the TcR-γ-chain may combine with the TcR-β-chain and in this way may play a role in T cell differentiation (17,18).

On the cell surface membrane the TcR is closely associated with the T cell antigen CD3 (4,5,30,31), which in humans consists of at least three proteins (CD3-γ, CD3-δ, and CD3-ϵ) (32,33). In the murine system a CD3-like protein complex has recently been identified (34,35). Cell surface expression of the TcR-$\alpha\beta$-CD3 complex seems to be dependent on the presence of the involved TcR protein chains (36,37). In this complex, the CD3 proteins may play a role in transduction of signals from TcR to cytoplasmic components (38,39). Recently the genes which code for the human CD3-δ- and CD3-ϵ-chain as well as their murine homologues have been identified (40-42). Transcription of these genes appears to be T cell-specific (15,40,42).

The precise order of gene rearrangement, transcription and expression of the TcR and CD3 genes during the very immature differentiation stages of human T cell development is not yet known. Since leukemias and non-Hodgkin lymphomas can be regarded as the malignant counterparts of normal hematopoietic cells (43-45), we analyzed 22 T cell acute lymphoblastic leukemias (T-ALL) to study the expression of the TcR-CD3 complex during early T cell differentiation. Ten of these T-ALL were arrested in very early stages of T cell differentiation, including three T-ALL which expressed the immunologic phenotype of the putative human prothymocyte (46).

MATERIALS AND METHODS

Cell samples

Bone marrow samples (n=7), peripheral blood samples (n=14) and a pleural exudate sample (n=1) were obtained from 22 different T- ALL patients. Eleven of these patients were children ($\leq$ 16 y) and the other eleven were adults (> 16 y). Mononuclear cells (MNC) were isolated from bone marrow and peripheral blood by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All washings were performed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), pH 7.8.

Immunologic marker analysis

The MNC were analyzed for the expression of terminal deoxynucleotidyl transferase (TdT), HLA-DR antigen, T cell markers CD7(Tp41), CD2(T11), CD5(T1), CD1(T6), CD4(T4), CD8(T8) and CD3(T3), and CD10 (common ALL antigen)(47-62). The CD2(T11) antigen, which represents the sheep red blood cell receptor, was detected by use of monoclonal antibodies (McAb) or by use of the E rosette test (54,55,63). In addition, the majority of the cell samples was characterized by use of the following McAb: the anti-B cell antigen McAb BA-1(CD24), BA-2(CD9) and B1(CD20) and the anti-myelomonocytic antigen McAb My9(CD33), VIM-D5(CD15), and My4(CD14) (64-69). The general characteristics of the antibodies used for the immunologic characterization are summarized in Table 1.

The reactivity of the antibodies was visualized by use of a fluorescein isothiocyanate-conjugated goat anti-rabbit-Ig antiserum (Supertechs, Bethesda, MD) in the case of the rabbit anti-TdT antiserum and a fluorescein isothiocyanate-conjugated goat anti-mouse-Ig antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) in the case of a mouse McAb. If more than 20% of MNC expressed a certain differentiation antigen the T-ALL was considered to be positive for this antigen.

Southern blot analysis

DNA was prepared from frozen MNC of the bone marrow, peripheral blood and pleural exudate samples, as described (70). From all T-ALL cell samples with the exception of the T-ALL sample from patient 1 enough DNA

TABLE 1. Antibodies used for the immunologic marker analysis of T-ALL cells.

Antibody	Cluster of differentiation[a]	Antigen recognized	References
Anti-TdT[b]	–	TdT	47
Anti-HLA-DR[c] or OKIa[d]	–	HLA-DR, non polymorphic antigen (gp29/34)	48, 49
3A1[e] or WT1[f]	CD7	Tp41 antigen (gp41)	50-53
Leu-5[c] or OKT11[d]	CD2	T11 antigen (gp50), sheep red blood cell receptor[g]	50, 54, 55
Leu-1[c] or OKT1[d]	CD5	T1 antigen (gp67)	50, 56, 57
Leu6[c] or OKT6[d] or 66IIC7[h]	CD1	T6 antigen (common thymocyte antigen)(gp43, gp45, gp49)	50, 58
Leu-3[c] or OKT4[d]	CD4	T4 antigen (gp60)	50, 59
Leu-2[c] or OKT8[d]	CD8	T8 antigen (gp32)	50, 59
Leu-4[c] or OKT3[d]	CD3	T3 antigen (gp16-25)	50, 60
VIL-A1[i] or J5[d]	CD10	common ALL antigen (gp100)	61, 62
BA-1[j]	CD24	B cell antigen (gp42)	64
BA-2[j]	CD9	B cell antigen (p24)	65
B1[k]	CD20	B cell antigen (p35)	66
My9[l]	CD33	myelo-monocytic antigen (gp67)	67
VIM-D5[i]	CD15	myeloid antigen	68
My4[l]	CD14	monocytic antigen (gp55)	69

a. As proposed by the Workshops on Human Leukocyte Differentiation Antigens (Paris, France, 1982; Boston, MA, 1984; Oxford, UK, 1986).
b. Supertechs, Bethesda, MD.
c. Becton Dickinson, Sunnyvale, CA.
d. Ortho Diagnostic Systems, Raritan, NJ.
e. American Type Culture Collection, Rockville, MD.
f. Dr. W. Tax, Nijmegen, The Netherlands.
g. The sheep red blood cell receptor (CD2 (T11) antigen) can also be demonstrated by use of the E rosette test.
h. Dr. J.M. van de Rijn, Amsterdam, The Netherlands.
i. Dr. W. Knapp, Vienna, Austria.
j. Hybritech, San Diego, CA.
k. Coulter Clone, Hialeah, FL.
l. Dr. J.D. Griffin, Boston, MA.

was obtained to study the configuration of the TcR-β genes as well as the TcR-γ genes. In the case of the T-ALL from patient 1 only the TcR-γ gene configuration was investigated. DNA (15 μg) was digested with *Eco*RI (New England Biolabs, Beverly, MA) and the fragments were separated by electrophoresis through a 0.7% agarose slab gel (Sigma Chemical Company, St. Louis, MO). DNA was transferred onto nitrocellulose as described (71). TcR-β gene and TcR-γ gene rearrangements were detected by hybridization with ^{32}P-labeled nick-translated probes.

Rearrangement in the β1 gene region was detected using the Cβ probe, while rearrangement in the β2 gene

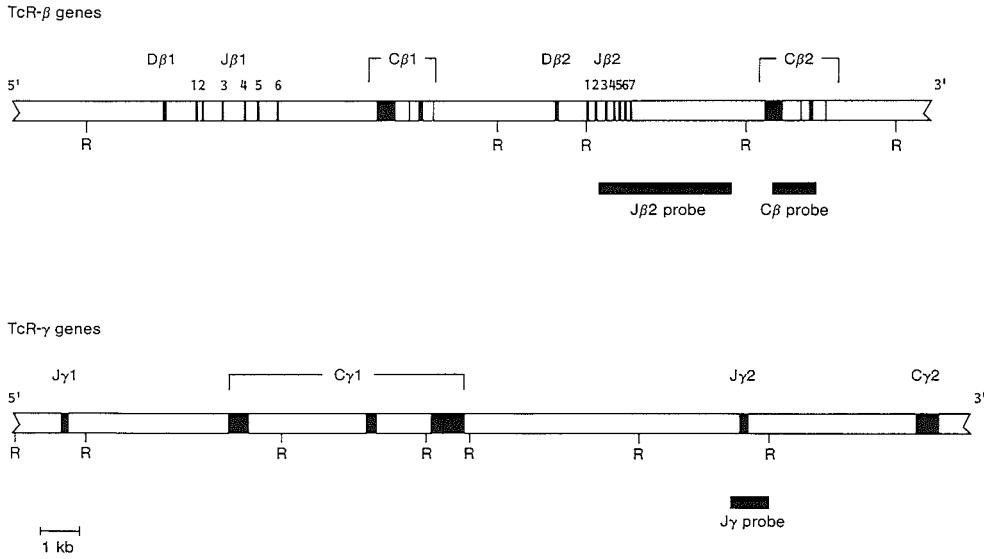


Figure 1. Organization of the joining, diversity and constant regions of the human TcR-β chain gene and the joining and constant regions of the human TcR-γ chain gene. The location of the relevant *Eco*RI (R) restriction enzyme sites are indicated. *Solid bars*, probes which were used for hybridization of the Southern blot filters. *Upper*, the Jβ2 probe consists of contiguous 1.9-kb *Pvu*II-*Pvu*II and 1.5-kb *Pvu*II-*Eco*RV fragments containing Jβ2.3 through Jβ2.7; this probe does not hybridize to the Jβ1 gene segment and was used to detect rearrangement in the β2 gene region. The Cβ probe is a 1.1-kb *Bgl*II-*Bgl*II fragment containing Cβ2 segments; this probe also hybridizes to the Cβ1 segment and was used to detect rearrangement in the β1 gene region. *Lower*, the Jγ probe is an 1.0-kb *Pst*I-*Eco*RI fragment containing Jγ2 segments; this probe hybridizes to both Jγ1 and Jγ2 gene segments and was used to detect rearrangements in these two gene regions.

region was detected using the Jβ2 probe. The Cβ probe is an 1.1-kb *Bgl*II-*Bgl*II fragment containing Cβ2 segments; it hybridizes to both Cβ1 and Cβ2 gene segments (Figure 1)(72). The Jβ2 probe consists of contiguous 1.9-kb *Pvu*II-*Pvu*II and 1.5-kb *Pvu*II-*Eco*RV fragments containing Jβ2.3 through Jβ2.7; this probe does not hybridize to the Jβ1 gene segment. (Figure 1)(72).

Rearrangements in the Jγ1 and Jγ2 gene regions were detected by use of the Jγ probe. The Jγ probe is a 1.0-kb *Pst*I-*Eco*RI fragment containing Jγ2 segments; it hybridizes to both Jγ1, and Jγ2 gene segments (Figure 1)(26). The isolation of probes, hybridization, and washing conditions were as described (73).

Northern blot analysis

Total RNA was isolated using the LiCl/urea method (71) from 12 of the 22 MNC samples (patients 1,3,7,10 to 12,14 to 16,19,21 and 22). In the case of the other 10 T-ALL samples no sufficient cell material was available to isolate total RNA. Approximately 15 μg of total RNA were size-fractionated on a 1.2% agarose gel in the presence of formaldehyde and blotted to Biodyne nylon membranes (Pall Ultrafine Filtration Corporation, Glen Core, NY). Specific RNA sequences were detected with ^{32}P-labeled nick-translated CD3 cDNA probes (CD3-δ:pPGBC9 (41); CD3-ϵ:pDJ1 (42)) and TcR cDNA probes (TcR-α:pA65 (74), TcR-β:pT10 (75); TcR-γ:pTγ-1 (26)). Hybridization and washing conditions were as described (73).

TABLE 2. Immunologic marker, Southern blot, and Northern blot analysis of the T-ALL cell samples[a].

	A (n=3)			B (n=7)							C (n=3)			D (n=4)				E (n=3)			F (n=2)	
Analysis	1 (PB)	2 (PB)	3 (BM)	4 (PB)	5 (PB)	6 (PB)	7 (BM)	8 (PB)	9 (BM)	10 (PB)	11 (BM)	12 (PE)	13 (BM)	14 (BM)	15 (BM)	16 (PB)	17 (PB)	18 (PB)	19 (PB)	20 (PB)	21 (PB)	22 (PB)
Immunologic marker[b]																						
TdT	90	84	92	90	90	100	96	87	84	96	74	97	92	92	81	99	95	81	95	47	0	0
HLA-DR	97	87	74	0	55	0	0	4	0	0	4	1	1	1	2	0	1	0	3	4	0	7
CD7 (Tp41)	92	96	85	95	98	100	99	85	99	99	97	98	96	94	95	99	90	89	95	90	100	75
CD2 (E/T11)[c]	98	91	87	36	98	57	83	40	9	80	96	95	78	95	94	79	99	96	99	92	99	88
CD5 (T1)	2	8	1	76	82	100	99	93	98	76	99	99	93	97	90	87	86	82	97	88	100	80
CD1 (T6)	0	0	0	0	0	0	0	1	9	4	93	98	92	25	52	45	23	0	1	1	0	0
CD4 (T4)	1	1	2	5	0	3	0	3	2	87	14	41	87	36	65	66	42	48	4	70	99	1
CD8 (T8)	2	3	1	4	0	2	0	5	1	57	21	70	90	3	66	11	52	73	70	68	92	80
CD3 (T3)	2	3	2	10	0	0	1	1	2	6	2	1	3	27	71	99	82	31	77	23	96	87
Southern blotting[d]																						
TcR-β1 genes		G	G	G	G	R	D	R	D	R	R	D	D	R	R	D	R	R	R	R	R	R
TcR-β2 genes		G	G	G	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	G	R	R
TcR-γ genes	G	G	G	G	G	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Northern blotting[e]																						
CD3-δ mRNA	+		+				+			+	+	+		+	+	+			+		+	+
CD3-ϵ mRNA	+		+				+			+	+	+		+	+	+			+		+	+
TcR-α mRNA (1.7 kb)			–				–			–	–	–		–	–	–			+		+	
TcR-β mRNA (1.0 kb)			–				–			±	±	–		–	–	±			±		±	
TcR-β mRNA (1.1 kb)			+				–			–	–	–		+	–	–			–		–	
TcR-β mRNA (1.3 kb)			–				–			+	+	+		–	+	+			+		+	
TcR-γ mRNA (1.6 kb)			–				+			±	–			+	–	+			±		±	

a. Abbreviations used in this table: PB, peripheral blood; BM, bone marrow; PE, pleural exudate.

b. The figures represent percentages of positive MNC.

c. The CD2 (T11) antigen (sheep red blood cell receptor) can be detected by use of McAb or by use of the E rosette test. In the case of patients 6 and 12 only the E rosette test was used. For the other patients the figures are given from the immunofluorescence analysis.

d. G, only germline bands were detected; R, at least one rearranged band was detected; D, neither rearranged bands nor the 10.5-kb germline band could be detected (i.e., both Cβ1 gene alleles were deleted).

e. +, normal or strong band; ±, weak band; –, no band detectable.

RESULTS

Immunologic marker analysis

The immunologic phenotypes of the 22 T-ALL (i.e. their expression of TdT, HLA-DR and the T cell markers CD7(Tp41), CD2(T11), CD5(T1), CD1(T6), CD4(T4), CD8(T8) and CD3(T3)) are summarized in Table 2. The T cell leukemias could be divided in three main groups, according to the expression of CD1 and CD3 : 10 immature T-ALL ($CD1^-/CD3^-$), seven common thymocytic T-ALL ($CD1^+/CD3^{-\text{ or }+}$), and five mature T-ALL ($CD1^-/CD3^+$). Each of the three main groups could be divided in two subgroups, based on the expression of TdT, CD7, CD5, CD1 and CD3, resulting in six different groups of T-ALL: A: three prothymocytic T-ALL ($TdT^+/CD7^+/CD5^-/CD1^-/CD3^-$); B: seven immature thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^-/CD3^-$); C: three $CD3^-$ common thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^+/CD3^-$); D: four $CD3^+$ common thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^+/CD3^+$); E: three TdT^+ mature T-ALL ($TdT^+/CD7^+/CD5^+/CD1^-/CD3^+$); and F: two TdT^- mature T-ALL ($TdT^-/CD7^+/CD5^+/CD1^-/CD3^+$) (Table 2 and Figure 7). Four T-ALL expressed HLA-DR; all three prothymocytic T-ALL and one immature thymocytic T-ALL (patient 5) (Table 2). All 22 T-ALL expressed the CD7 antigen as well as the CD2 antigen, except one immature thymocytic T-ALL (patient 9), which did not express the CD2 antigen (Table 2).

CD10 (common ALL) antigen was weakly expressed by 9 of the 22 T-ALL: patients 4, 6, 8, 10, 12, 13, 14, 16, and 18. Also the CD24(BA-1) antigen was weakly expressed by some T-ALL (patients 8, 16, and 21). Weak expression of B cell markers such as CD10 or CD24 is often seen in T-ALL (76). The B cell antigens CD9(BA-2) and CD20(B1) and the myelomonocytic antigens CD33(My9), CD15(VIM-D5), and CD14(My4) were not expressed by the T-ALL tested (data not shown).

Southern blot analysis

To detect TcR-β gene rearrangements in the 21 T-ALL tested, the *Eco*RI digested DNAs were analyzed by use of the TcR-β probes Cβ and Jβ2 (Figure 1). Hybridization with the Cβ probe resulted in germline bands of about 3.8 kb (Cβ2 gene segment) and 10.5 kb (Cβ1 gene segment) in the two prothymocytic T-ALL tested (patients 2 and 3) as well as in two immature thymocytic T-ALL (patients 4 and 5). In all other patients at least one rearranged band was detected or only the 3.8-kb germline band was detected; the latter indicates that the Cβ1 gene segments on both chromosomes are deleted (Table 2 and Figure 2). Hybridization with the Jβ2 probe resulted in the germline band of about 4.1 kb in the two prothymocytic T-ALL tested (patients 2 and 3) and in one immature thymocytic T-ALL (patient 4), while in all other patients at least one rearranged band was detected, except in patient 20. (Table 2 and Figure 3). These data indicate that in patients 2, 3 and 4 the TcR-β genes were in germline configuration, while in all other patients the TcR-β genes were rearranged.

To detect TcR-γ gene rearrangements in the 22 T-ALL cell samples, the Southern blot filters were hybridized with the Jγ probe (Figure 1). In all three prothymocytic T-ALL (patients 1, 2 and 3) as well as in two immature thymocytic T-ALL (patients 4 and 5) only germline bands of 1.8

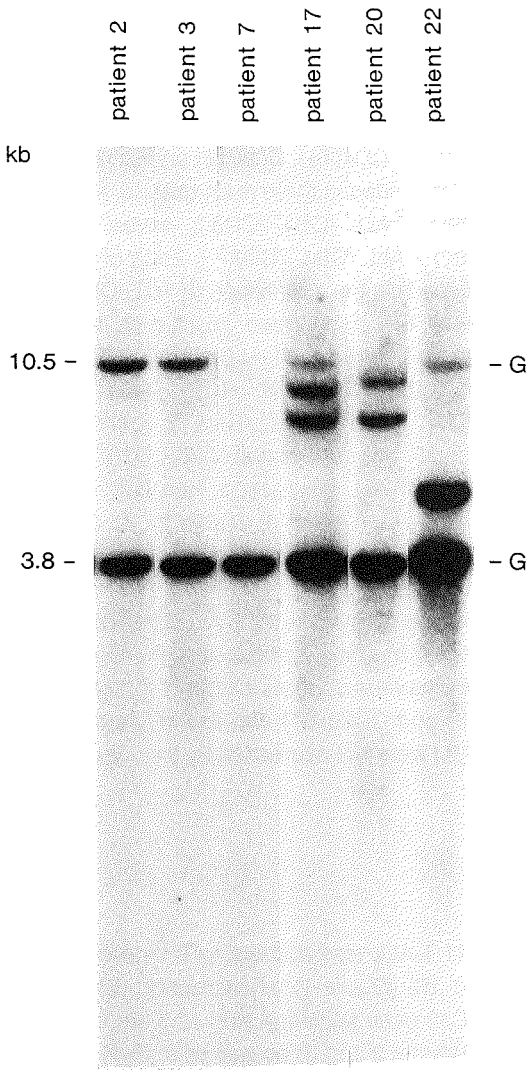


Figure 2. Southern blot analysis of *Eco*RI-digested DNA for the detection of rearrangement in the $\beta 1$ gene region, by use of the $C\beta$ probe. The two germline bands (G) of about 10.5 kb ($C\beta 1$ gene segment) and 3.8 kb ($C\beta 2$ gene segment) are indicated. The ALL cells from patients 2 and 3 had germline TcR-$\beta 1$ genes, while in the ALL cells from patients 7, 17, 20 and 22 the TcR-$\beta 1$ genes appeared to be clonally rearranged or deleted. The weak 10.5-kb germline band in the lanes from patients 17 and 22 represents DNA from contaminating normal MNC.

kb ($J\gamma 1$ gene segment) and 3.3 kb ($J\gamma 2$ gene segment) were present. In all other T-ALL at least one rearranged band was detected. In six T-ALL, especially mature T-ALL, one rearranged band was present, while in the remaining 11 T-ALL at least two rearranged bands were present (Table 2 and Figure 4). In addition to the six different types of rearrangements detected with the $J\gamma$ probe, recently described in the classification of rearranged TcR-γ genes (26), we did detect an additional type of TcR-γ gene rearrangement, i.e. a non-germline band of 2.3 kb (Figure 4). The $J\gamma$ probe detects rearrangement in the two highly homologous $J\gamma 1$ and $J\gamma 2$ gene regions, initially described (26,27). Recent work indicates that additional diversity may be afforded to the TcR-γ chain by additional joining regions in association with the two described

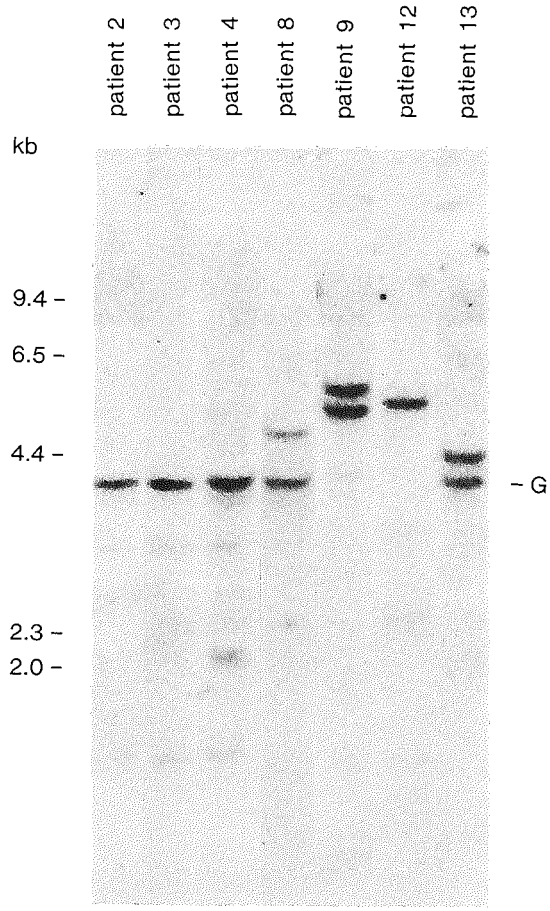


Figure 3. Southern blot analysis of *Eco*RI-digested DNA for the detection of rearrangement in the $\beta 2$ gene region by use of the $J\beta 2$ probe. The 4.1-kb germline band (G) is indicated on the right, while the size of the used molecular weight markers is indicated on the left. The T-ALL cells from patients 2, 3, and 4 had germline TcR-$\beta 2$ genes, while in the ALL cells from patients 8, 9, 12, and 13 one or two alleles of the TcR-$\beta 2$ genes were clonally rearranged or deleted.

constant regions (77)(unpublished results). Rearrangements to these joining segments has not yet been characterized. It is possible that cells showing a germline configuration with the Jγ probe may actually be rearranged to these other regions. At any rate, the Jγ probe detects a difference between the prothymocytic leukemias and two immature thymocytic T-ALL (patients 1 to 5) and the other T-ALL (patients 6 to 22). Either the prothymocytic leukemias and the two immature thymocytic T-ALL are truly unrearranged or are rearranged to joining regions not commonly utilized by more mature T cells.

Northern blot analysis

To analyze the expression of genes encoding for protein chains of the TcR-CD3 complex, we performed Northern blot analysis on RNA from 12 out of the 22 T-ALL by using the described TcR and CD3 cDNA probes.

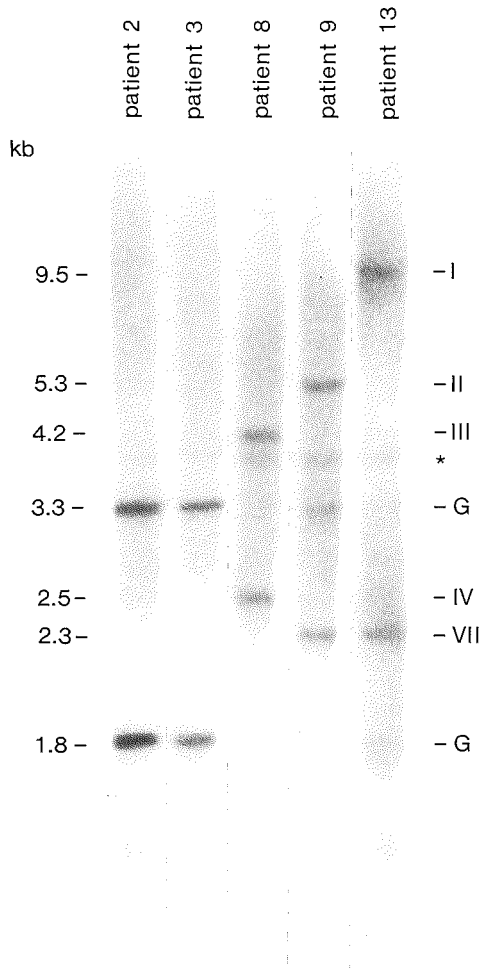


Figure 4. Southern blot analysis of *Eco*RI-digested DNA for the detection of TcR-γ gene rearrangements using the Jγ probe. The two germline bands (G) of about 1.8 kb (Jγ1 gene segment) and 3.5 kb (Jγ2 gene segment) are indicated. The weak 3.8-kb bands (*), present in all five lanes, are old hybridization signals from a previous hybridization. The T-ALL cells from patient 2 and 3 had germline Jγ1 and Jγ2 gene regions, while in the ALL cells from patients 8, 9 and 13 the TcR-γ genes appeared to be clonally rearranged. In the lanes from patients 8, 9 and 13 weak germline bands are also present. The non-germline bands are marked with Roman figures, according to the previously described classification of rearranged TcR-γ genes (26) and their molecular weight is indicated on the left. The non-germline 2.3-kb band (VII) represents a new group of rearranged TcR-γ genes.

Hybridization with the CD3-δ and CD3-ε probes revealed that normal size transcripts of the CD3-δ and CD3-ε genes were present in all 12 T-ALL tested, including the two tested prothymocytic T-ALL and four additional $CD3^-$ T-ALL (Table 2 and Figure 5).

Hybridization with the TcR-α cDNA probe revealed that the two mature T-ALL tested expressed TcR-α transcripts, while in all other T-ALL tested no TcR-α transcripts could be detected, including the three $CD3^+$ common thymocytic T-ALL tested (Table 2).

The TcR-β cDNA probe detected a 1.3-kb mRNA as well as a 1.0-kb mRNA in several leukemias. The 1.3-kb mRNA, which represents a complete variable-diversity-joining constant transcript (78,79), was present in one immature thymocytic T-ALL (patient 10) and in all common thymocytic and mature T-ALL tested, except one (patient 14). The 1.0-kb mRNA, which may represent a diversity-joining-constant transcript (79), was present in the majority of T-ALL which contained 1.3-kb TcR-β mRNA. Patient 14 had a $CD3^+$ T-ALL, which only expressed

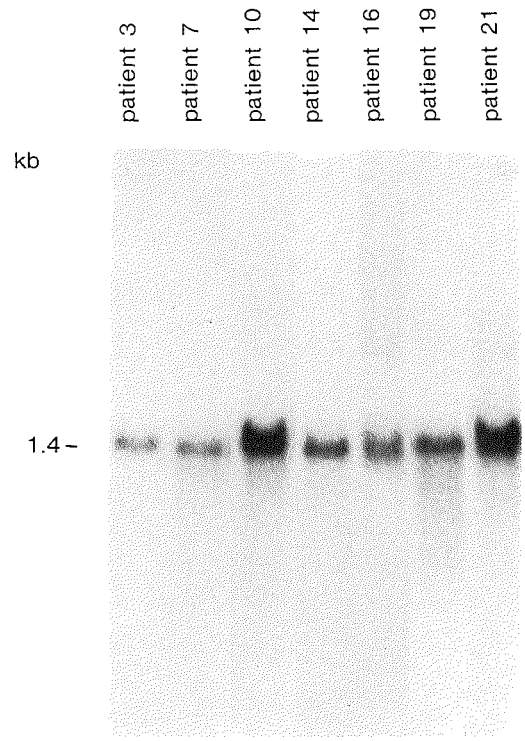


Figure 5. Northern blot analysis for the detection of CD3-ϵ transcripts. The size of the CD3-ϵ transcript (1.4 kb) is indicated. The T-ALL cells from all patients appeared to contain CD3-ϵ transcripts.

intermediate sized TcR-β transcripts of about 1.1 kb. This 1.1-kb band was also present in a prothymocytic T-ALL (patient 3), which had germline TcR-β chain genes (Table 2 and Figure 6).

Hybridization with the TcR-γ cDNA probe revealed that distinct TcR-γ mRNA was present in several immature and common thymocytic T-ALL, but was very weakly expressed by the mature T-ALL tested. The prothymocytic T-ALL tested did not contain TcR-γ mRNA (Table 2).

DISCUSSION

The T cell leukemias studied here could be divided into six groups based on their immunologic phenotype. These six groups of T-ALL represent the various stages of T cell differentiation: A: prothymocytic T-ALL ($TdT^+/CD7^+/CD5^-/CD1^-/CD3^-$); B: immature thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^-/CD3^-$); C: $CD3^-$ common thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^+/CD3^-$); D: $CD3^+$ common thymocytic T-ALL ($TdT^+/CD7^+/CD5^+/CD1^+/CD3^+$); E: TdT^+ mature T-ALL ($TdT^+/CD7^+/CD5^+/CD1^-/CD3^+$); and F: TdT^- mature T-ALL ($TdT^-/CD7^+/CD5^+/CD1^-/CD3^+$) (Table 2 and Figure 7).

In the prothymocytic T-ALL the TcR-β genes were in germline configuration, as were the Jγ1 and Jγ2 segments of the TcR-γ genes. Two immature thymocytic T-ALL also had germline

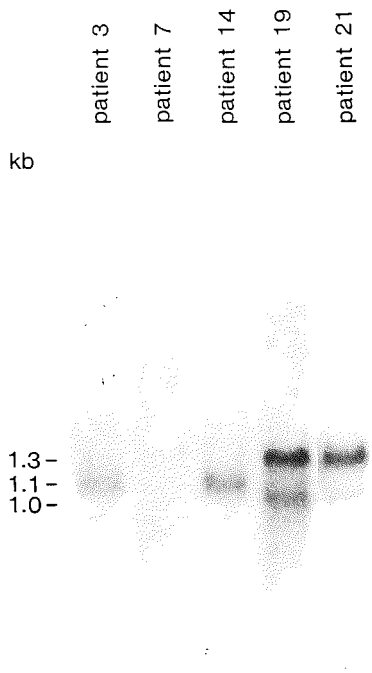


Figure 6. Northern blot analysis for the detection of TcR-β transcripts. The sizes of the TcR-β transcripts (1.3 kb, 1.1 kb, and 1.0 kb) are indicated. The presumptive functional 1.3-kb TcR-β transcripts as well as weak bands of 1.0-kb transcripts were detected in patients 19 and 21. In patients 3 and 14, only intermediated sized 1.1-kb transcripts were detected, while in patient 7, no TcR-β transcripts could be detected.

TcR-γ genes. This is remarkable, since virtually all T-ALL reported until now had rearranged TcR-γ genes (24-26). One of the two immature thymocytic T-ALL with the germline TcR-γ genes (patient 4) appeared to have also germline TcR-β genes. In the other one (patient 5) germline bands were detected by use of the Cβ probe, while one non-germline band was detected by use of the Jβ2 probe, suggesting a diversity-joining rearrangement in the β2 region. It may be possible that in this leukemia the TcR-γ genes are not germline, but rearranged to one of the recently described joining regions (77). In all other T-ALL (n=17) the TcR-γ genes as well as the TcR-β genes were rearranged. These data indicate that rearrangement of TcR-γ and TcR-β genes takes place in early stages of thymocytic T cell differentiation, while prothymocytes still have germline TcR-γ and TcR-β genes.

The two tested prothymocytic T-ALL as well as all other T-ALL tested contained CD3-δ and CD3-ϵ transcripts. This finding supports the T cell origin of the prothymocytic T-ALL, since the presence of CD3 mRNA and CD3 proteins is T cell specific (41,42,60,80). On the other hand the presence of germline TcR-β and TcR-γ genes strongly supports the very immature character of the prothymocytic T-ALL, i.e. the expression of TdT, HLA-DR, and the two pan-T cell antigens CD7 and CD2, but the absence of all other cell surface T cell markers tested. Although all HLA-DR$^+$ ALL reported until now, appeared to have rearranged Ig heavy chain genes (16,81-83), the two prothymocytic T-ALL tested (patients 1 and 3) had germline Ig heavy chain genes (data not shown). Previously we have described the immunologic phenotype of the normal counterpart of the prothymocytic T-ALL and reported that this cell is detectable in low frequen-

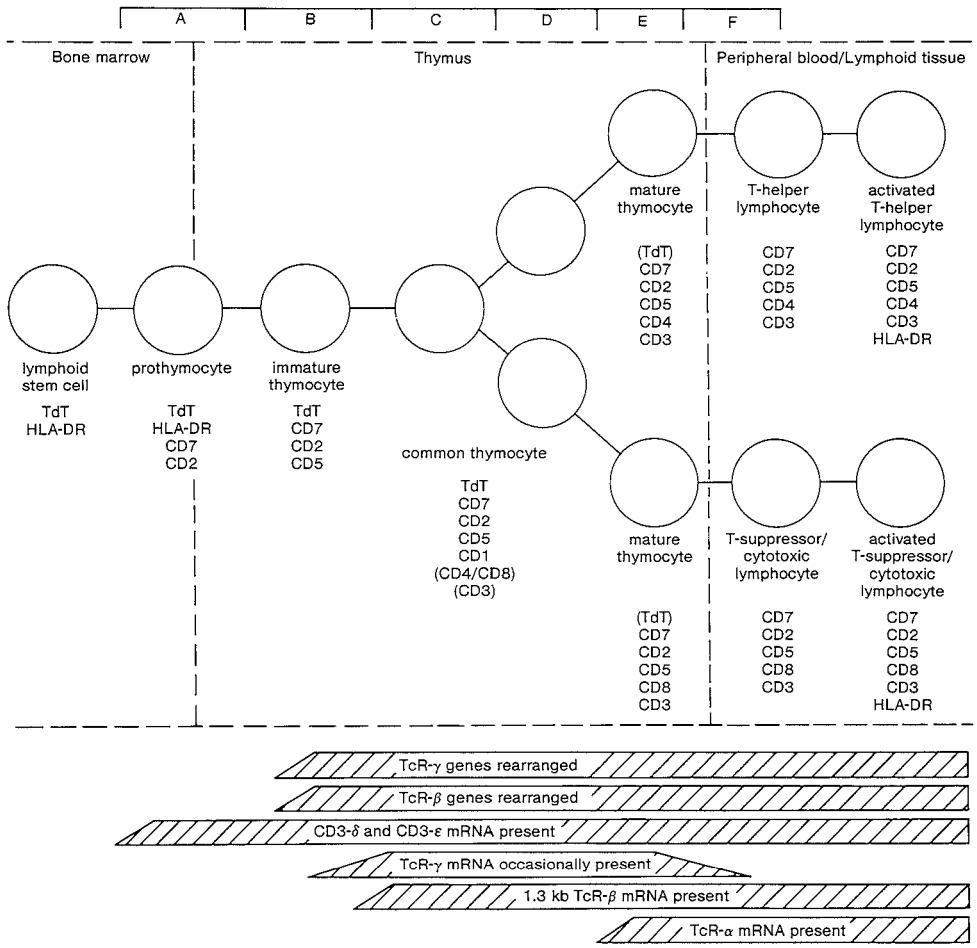


Figure 7. Hypothetical differentiation scheme of the human T cell lineage. The immunologic phenotypes of the cells in the various T cell differentiation stages are indicated. A, B, C, D, E, and F, six groups of T-ALL used in this study. Dotted lines, the tissues where these cells are found; horizontal bars, the described data concerning the rearrangement of the TcR-γ and TcR-β genes as well as the presence of transcripts for CD3-δ, CD3-ε, TcR-α, TcR-β, and TcR-γ genes. This differentiation scheme is hypothetical and does not pretend to be complete. In addition, future experiments have to elucidate whether $CD3^+$ cells, which contain TcR-γ transcripts but lack TcR-α transcripts, belong to a distinct differentiation lineage.

cies in the bone marrow (0.02% to 0.05%) as well as the thymus (0.02%) (46). Occasionally this putative prothymocyte can be detected in the peripheral blood (46,84), suggesting migration from the bone marrow via the peripheral blood to the thymus. All these data may indicate that CD3 gene transcription is an early event in T cell differentiation, which most probably already takes place before entrance into the thymus, while TcR-β and TcR-γ gene rearrangements may be thymus-restricted processes (Figure 7).

It is remarkable that HLA-DR positivity was only found in immature T-ALL with germline

TcR-β genes and/or germline TcR-γ genes: all prothymocytic T-ALL were HLA-DR$^+$ and half of the leukemic cells of one of the immature thymocytic T-ALL (patient 5) were HLA-DR$^+$. This is in line with the observation that most precursor cells in hematopoiesis express the HLA-DR antigen (85), and suggests that the expression of HLA-DR together with CD3 gene expression and the expression of the pan-T cell markers CD7 and CD2 may play a role in the initial phase of T cell development.

Data from the literature suggest that in mice, TcR-γ genes are expressed prior to TcR-β genes during ontogeny (17,18). Our data indicate that in humans TcR-γ and TcR-β genes rearrange in virtually the same early thymocytic differentiation stage. Nevertheless, TcR-γ transcripts were detectable in an earlier stage than 1.3-kb TcR-β transcripts. Although ontogeny and differentiation are not completely comparable, these data suggest that T cell development in the murine and human species is rather similar.

Preliminary data indicate that in humans as well as in mice the repertoire of the TcR-γ genes is restricted as compared with the TcR-α and TcR-β gene repertoire (23,26,86). In addition to the recently described classification of TcR-γ gene rearrangements, we here report an additional type of rearranged TcR-γ genes, which results in a 2.3-kb band (Figure 4) (*Eco*RI digestion and hybridization with the Jγ probe); we propose to use the Roman number VII (Figure 4), for this type of TcR-γ gene rearrangement (26). Future experiments will elucidate the extent of the TcR-γ gene repertoire and will demonstrate whether the recently discovered TcR-γ joining regions are used in prothymocytic T-ALL.

In mice the presence of TcR-γ transcripts is restricted to thymocytic cells (17). Our human data are similar, since distinct levels of TcR-γ transcripts were only present in some thymocytic T-ALL, while the mature T- ALL were weakly positive. In the tested prothymocytic T-ALL (patient 3) no TcR-γ transcripts could be detected, which was in line with the presence of germline TcR-γ genes, as analyzed by use of the described Jγ probe.

In many tested T-ALL two TcR-β transcripts of different sizes were detected (1.3 kb and 1.0 kb). The presumptive functional 1.3-kb TcR-β transcript, which represents a complete variable-diversity-joining-constant transcript (78,79), was detected in almost all CD3$^-$ and CD3$^+$ common thymocytic T-ALL as well as mature T-ALL tested. In most of these T-ALL 1.0-kb TcR-β transcripts were also detected. Such transcripts most probably represent diversity-joining-constant transcripts (79). One CD3$^+$ common thymocytic T-ALL (patient 14) only expressed an intermediate-sized 1.1-kb TcR-β transcript and no detectable 1.3- or 1.0-kb transcripts. Since it has been suggested that expression of CD3 on the cell surface requires the presence of TcR chains (36,37), it is possible that an incomplete TcR-β-chain may be present in the TcR-CD3 complex expressed by this T-ALL or that alternatively a non-$\alpha\beta$ TcR is expressed (29). In the tested prothymocytic T-ALL (patient 3) also a weak intermediate-sized 1.1-kb TcR-β transcript was detected, while all TcR-β-chain genes in this leukemia were in germline configuration. This suggests that the 1.1-kb transcript may represent an aberrant joining-constant or constant transcript. In one of the immature thymocytic T-ALL (patient 7) no TcR-β transcripts were detectable, although the TcR-β genes were rearranged. In the latter leukemia rearrangement of the TcR-β genes may have resulted in a transcriptional inactive gene, or alternatively this leukemia may represent a cell in an intermediate differentiation stage between rearrangement and transcription of the TcR-β genes.

The TcR-α transcripts were detected in mature T-ALL, but not in the CD3$^+$ common

thymocytic T-ALL tested. Since it has been suggested that CD3 expression is dependent on the presence of TcR chains, this may indicate that the level of TcR-α mRNA is below the detection limit, or that the TcR-α chain is replaced by another chain, e.g. TcR-γ (17). This presumption is supported by the presence of TcR-γ transcripts in two of the three $CD3^+$ common thymocytic T-ALL tested (Table 2). An alternate non-αβ TcR has recently been described by Brenner et al. (29). Such non-αβ TcR may be expressed by the T-ALL from patient 14, in which TcR-γ mRNA was detected in absence of TcR-α mRNA and 1.3-kb TcR-β mRNA.

Summarizing, we conclude that the presence of CD3 transcripts but absence of TcR gene rearrangements as well as Ig gene rearrangements in TdT^+/HLA-DR^+/$CD7^+$/$CD2^+$/$CD5^-$/$CD1^-$/$CD3^-$ T-ALL strongly supports our hypothesis that the normal counterparts of such leukemic cells in human bone marrow represent prothymocytes. On the other hand, this indicates that CD3 gene transcription is one of the earliest events in T cell differentiation, which already takes place in prothymocytes, most probably before entrance into the thymus. The TcR-γ and TcR-β genes rearrange in a subsequent stage of T cell differentiation (i.e. early in thymic T cell differentiation) and can consequently be transcribed. The TcR-γ genes may only be transcribed transiently during thymic T cell differentiation, while the TcR-β transcription continues during further differentiation. Subsequently the TcR-α genes are transcribed. The latter event may be the final step in the production of the complete set of TcR and CD3 proteins, resulting in the expression of the TcR-αβ-CD3 complex at the cell surface of mature T cells (Figure 7). The consecutive processes described here are very analogous to those that precede the surface membrane Ig expression during B cell differentiation. Recently Furley et al. have published similar data (87). However in their series of T-ALL no prothymocytic T-ALL were included and they did not analyze the configuration and expression of the TcR-γ genes.

Whether the $CD3^+$ common thymocytic T-ALL, which lack TcR-α transcripts, express an alternate receptor such as TcR-βγ or another TcR and whether the normal counterparts of these T-ALL may belong to a distinct differentiation lineage has to be further investigated.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof. Dr. R. Benner for his continuous support and advice, Mr. C.J. van Dijk (Audiovisueel Centrum of the Erasmus University) and Mr. T.M. van Os for their excellent photographic assistance and Mrs. J. de Goey-van Dooren and Mrs. G. de Korte for their skillful secretarial support.

This work was supported by the Netherlands Cancer Foundation (J.J.M van Dongen and I.L.M. Wolvers-Tettero), the Sophia Foundation for Medical Research (H.J. Adriaansen), the Deutsche Forschungsgemeinschaft (C.R. Bartram), the Medical Research Council of Canada (A.D. Duby) and the Charles A. King Trust of Boston (P. van den Elsen).

REFERENCES

1. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
2. Marrack P, Kappler J. The T cell and its receptor. Sci Am 1986;254:36-45.
3. Acuto O, Fabbi M, Bensussan A, Milanese C, Campen TJ, Royer HD, Reinherz EL. The human T-cell receptor. J Clin Immunol 1985;5:141-57.
4. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen specific human T cell function. J Exp Med 1983;157:705-19.

5. Acuto O, Meuer SC, Hodgdon JC, Schlossman SF, Reinherz EL. Peptide variability exists within α and β subunits of the T cell receptor for antigen. J Exp Med 1983;158:1368-73.
6. Hedrick SM, Nielsen EA, Kavaler J, Cohen DI, Davis MM. Sequence relationships between putative T-cell receptor polypeptides and immunoglobulins. Nature 1984;308:153-8.
7. Yanagi Y, Yoshikai Y, Leggett K, Clark SP, Aleksander I, Mak TW. A human T cell-specific cDNA clone encodes a protein having extensive homology to immunoglobulin chains. Nature 1984;308:145-9.
8. Chien Y-h, Gascoigne NRJ, Kavaler J, Lee NE, Davis MM. Somatic recombination in a murine T-cell receptor gene. Nature 1984;309:322-6.
9. Siu G, Clark SP, Yoshikai Y, Malissen M, Yanagi Y, Strauss E, Mak TW, Hood L. The human T cell antigen receptor is encoded by variable, diversity, and joining gene segments that rearrange to generate a complete V gene. Cell 1984;37:393-401.
10. Hannum CH, Kappler JW, Trowbridge IS, Marrack P, Freed JH. Immunoglobulin-like nature of the α-chain of a human T-cell antigen/MHC receptor. Nature 1984;312:65-7.
11. Fabbi M, Acuto O, Smart JE, Reinherz EL. Homology of Tiα-subunit of a T cell antigen-MHC receptor with immunoglobulin. Nature 1984;312:269-71.
12. Hood L, Kronenberg M, Hunkapiller T. T cell antigen receptors and the immunoglobulin supergene family. Cell 1985;40:225-9.
13. Royer HD, Acuto O, Fabbi M, Tizard R, Ramachandran K, Smart JE, Reinherz EL. Genes encoding the Tiβ subunit of the antigen/MHC receptor undergo rearrangement during intrathymic ontogeny prior to surface T3-Ti expression. Cell 1984;39:261-6.
14. Born W, Yagüe J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor β-chain genes during T-cell development. Proc Natl Acad Sci USA 1985;82:2925-9.
15. Samelson LE, Lindsten T, Fowlkes BJ, Van den Elsen P, Terhorst C, Davis MM, Germain RN, Schwartz RH. Expression of genes of the T-cell antigen receptor complex in precursor thymocytes. Nature 1985;315:765-8.
16. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
17. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.
18. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
19. Collins MKL, Tanigawa G, Kissonerghis A-M, Ritter M, Price KM, Tonegawa S, Owen MJ. Regulation of T-cell receptor gene expression in human T-cell development. Proc Natl Acad Sci USA 1985;82:4503-7.
20. Royer HD, Ramarli D, Acuto O, Campen TJ, Reinherz EL. Genes encoding the T-cell receptor α and β subunits are transcribed in an ordered manner during intrathymic ontogeny. Proc Natl Acad Sci USA 1985;82:5510-4.
21. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 1984;309:757-62.
22. Hayday AC, Saito H, Gillies SD, Kranz DM, Tanigawa G, Eisen HN, Tonegawa S. Structure, organization, and somatic rearrangement of T cell gamma genes. Cell 1985;40:259-69.
23. Kranz DM, Saito H, Heller M, Takagaki Y, Haas W, Eisen HN, Tonegawa S. Limited diversity of the rearranged T-cell γ gene. Nature 1985;313:752-5.
24. Lefranc M-P, Rabbitts TH. Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types. Nature 1985;316:464-6.
25. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. Nature 1985;316:549-52.
26. Quertermous T, Murre C, Dialynas D, Duby AD, Strominger JL, Waldmann TA, Seidman JG. Human T-cell γ chain genes: organization, diversity, and rearrangement. Science 1986;231:252-5.
27. Lefranc M-P, Forster A, Rabbitts TH. Rearrangement of two distinct T-cell γ-chain variable-region genes in human DNA. Nature 1986;319:420-2.

28. Dialynas DP, Murre C, Quertermous T, Boss JM, Leiden JM, Seidman JG, Strominger JL. Cloning and sequence analysis of complementary DNA encoding an aberrantly rearranged human T-cell γ chain. Proc Natl Acad Sci USA 1986;83:2619-23.
29. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
30. Reinherz EL, Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Acuto O, Schlossman SF. Comparison of T3-associated 49- and 43-kilodalton cell surface molecules on individual human T-cell clones: evidence for peptide variability in T-cell receptor structures. Proc Natl Acad Sci USA 1983;80:4104-8.
31. Brenner MB, Trowbridge IS, Strominger JL. Crosslinking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. Cell 1985;40:183-90.
32. Oettgen HC, Kappler J, Tax WJM, Terhorst C. Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. J Biol Chem 1984;259:12039-48.
33. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The δ- and ϵ-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature 1984;312:455-8.
34. Allison JP, Lanier LL. Identification of antigen receptor-associated structures on murine T cells. Nature 1985;314:107-9.
35. Oettgen HC, Pettey CL, Maloy WL, Terhorst C. A T3-like protein complex associated with the antigen receptor on murine T cells. Nature 1986;320:272-5.
36. Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, Calman AF, Terhorst C, Stobo JD, Weiss A. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. Nature 1985;316:606-9.
37. Van Dongen JJM, Geurts van Kessel AHM, Wolvers-Tettero ILM, Versnel MA, Van Oudenaren A, Schoenmaker E, Hagemeijer A. Construction of human-mouse T cell hybrids that express human T cell-associated surface antigens and allow the chromosomal localization of these antigens. J Immunol 1986;137:1047-53.
38. Owen MJ. T-cell receptor companions. Nature 1984;312:406.
39. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, Stobo J. The role of the T3/antigen receptor complex in T-cell activation. Annu Rev Immunol 1986;4:593-619.
40. Van den Elsen P, Shepley B-A, Borst J, Coligan JE, Markham AF, Orkin S, Terhorst C. Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex. Nature 1984;312:413-8.
41. Van den Elsen P, Shepley B-A, Cho M, Terhorst C. Isolation and characterization of a cDNA clone encoding the murine homologue of the human 20K T3/T-cell receptor glycoprotein. Nature 1985;314:542-4.
42. Gold DP, Puck JM, Pettey CL, Cho M, Coligan J, Woody JN, Terhorst C. Isolation of cDNA clones encoding the 20K nonglycosylated polypeptide chain of the human T-cell receptor/T3 complex. Nature 1986;321:431-4.
43. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
44. Foon KA, Schroff RW, Gale RP. Surface markers on leukemia and lymphoma cells: recent advances. Blood 1982;60:1-19.
45. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
46. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
47. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
48. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:293-9.
49. Reinherz EL, Kung PC, Pesando JM, Ritz J, Goldstein G, Schlossman SF. Ia determinants on human T-cell subsets defined by monoclonal antibody: activation stimuli required for expression. J Exp Med 1979;150:1472-82.
50. Janossy G, Prentice HG. T cell subpopulations, monoclonal antibodies and their therapeutic applications. In: Janossy G, ed. Clinics in heamatology. London: Saunders Company 1982;11:631-60.
51. Haynes BF, Mann DL, Hemler ME, Schroer JA, Shelhamer JH, Eisenbarth GS, Strominger JL, Thomas CA, Mostowski HS, Fauci AS. Characterizaton of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans. Proc Natl Acad Sci USA 1980;77:2914-8.

52. Vodinelich L, Tax W, Bai Y, Pegram S, Capel P, Greaves MF. A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 1983;62:1108-13.
53. Sutherland DR, Rudd CE, Greaves MF. Isolation and characterization of a human T lymphocyte-associated glycoprotein (gp40). J Immunol 1984;133:327-33.
54. Howard FD, Ledbetter JA, Wong J, Bieber CP, Stinson EB, Herzenberg LA. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block E-rosette formation. J Immunol 1981;126:2117-22.
55. Verbi W, Greaves MF, Schneider C, Koubek K, Janossy G, Stein H, Kung P, Goldstein G. Monoclonal antibodies OKT11 and OKT11A have pan-T reactivity and block sheep erythrocyte "receptors". Eur J Immunol 1982;12:81-6.
56. Engleman EG, Warnke R, Fox RI, Dilley J, Benike CJ, Levy R. Studies of a human T lymphocyte antigen recognized by a monoclonal antibody. Proc Natl Acad Sci USA 1981:78:1791-5.
57. Martin PJ, Hansen JA, Siadak AW, Nowinski RC. Monoclonal antibodies recognizing normal human T lymphocytes and malignant human B lymphocytes: a comparative study. J Immunol 1981; 127:1920-3.
58. Lerch PG, Van de Rijn M, Schrier P, Terhorst C. Biochemical comparison of the T6 antigen and HLA-A,B antigens. Hum Immunol 1983;6:13-30.
59. Engleman EG, Benike CJ, Glickman E, Evans RL. Antibodies to membrane structures that distinguish suppressor/cytotoxic and helper T lymphocyte subpopulations block the mixed leukocyte reaction in man. J Exp Med 1981;153:193-8.
60. Borst J, Prendiville MA, Terhorst C. Complexity of the human T lymphocyte-specific cell surface antigen T3. J Immunol 1982;128:1560-5.
61. Knapp W, Majdic O, Bettelheim P, Liszka K. VIL-A1, a monoclonal antibody reactive with common acute lymphatic leukemia cells. Leuk Res 1982;6:137-47.
62. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF. A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 1980;283:583-5.
63. Moore AL, Zusman J. Conditions enhancing the percentage and size of E rosettes formed by T lymphocytes. J Immunol Methods 1978;23:275-84.
64. Abramson CS, Kersey JH, LeBien TW. A monoclonal antibody (BA-1) reactive with cells of human B lymphocyte lineage. J Immunol 1981;126:83-8.
65. Kersey JH, LeBien TW, Abramson CS, Newman R, Sutherland R, Greaves M. p24: A human leukemia-associated and lymphohemopoietic progenitor cell surface structure identified with monoclonal antibody. J Exp Med 1981;153:726-31.
66. Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterization of a human B lymphocyte-specific antigen. J Immunol 1980;125:1678-85.
67. Sabbath KD, Ball ED, Larcom P, Davis RB, Griffin JD. Heterogeneity of clonogenic cells in acute myeloblastic leukemia. J Clin Invest 1985;75:746-53.
68. Majdic O, Liszka K, Lutz D, Knapp W. Myeloid differentiation antigen defined by a monoclonal antibody. Blood 1981;58:1127-33.
69. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557-63.
70. Jeffreys AJ, Flavell RA. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell 1977;12:429-39.
71. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1982
72. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.
73. Bernards R, Flavell RA. Physical mapping of the globin gene deletion in hereditary persistence of foetal haemoglobin (HPFH). Nucleic Acids Res 1980;8:1521-34.
74. Sim GK, Yagüe J, Nelson J, Marrack P, Palmer E, Augustin A, Kappler J. Primary structure of human T-cell receptor α-chain. Nature 1984;312:771-5.
75. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a

human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-6.
76. Neudorf SML, LeBien TW, Kersey JH. Characterization of thymocytes expressing the common acute lymphoblastic leukemia antigen. Leuk Res 1984;8:173-9.
77. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
78. Kavaler J, Davis MM, Chien Y-h. Localization of a T-cell receptor diversity-region element. Nature 1984;310:421-3.
79. Siu G, Kronenberg M, Strauss E, Haars R, Mak TW, Hood L. The structure, rearrangement and expression of Dβ gene segments of the murine T-cell antigen receptor. Nature 1984;311:344-50.
80. Reinherz EL, Kung PC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA 1980;77:1588-92.
81. Ford AM, Molgaard HV, Greaves MF, Gould HJ. Immunoglobulin gene organisation and expression in haemopoietic stem cell leukaemia. EMBO J 1983;2:997-1001.
82. Bakhshi A, Minowada J, Arnold A, Cossman J, Jensen JP, Whang-Peng J, Waldmann TA, Korsmeyer SJ. Lymphoid blast crises of chronic myelogenous leukemia represent stages in the development of B-cell precursors. N Engl J Med 1983;309:826-31.
83. Ha K, Hozumi N, Hrincu A, Gelfand EW. Lineage specific classification of leukaemia: results of the analysis of sixty cases of childhood leukaemia. Brit J Haematol 1985;61:237-49.
84. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986;113-33.
85. Falkenburg JHF, Jansen J, Van der Vaart-Duinkerken N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ. Polymorphic and monomorphic HLA-DR determinants on human hematopoietic progenitor cells. Blood 1984;63:1125-32.
86. Garman RD, Doherty PJ, Raulet DH. Diversity, rearrangement, and expression of murine T cell gamma genes. Cell 1986;45:733-42.
87. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.

CHAPTER 4.4

HUMAN T CELL GAMMA CHAIN JOINING REGIONS AND T CELL DEVELOPMENT*

Thomas Quertermous[1], William M. Strauss[2], Jacques J.M. van Dongen[3] and J.G. Seidman[2]

1. Cardiac Unit, Department of Medicine, Massachusetts General Hospital, Boston, MA 02114;
2. Department of Genetics, Harvard Medical School, Boston, MA 02115;
3. Department of Immunology, Erasmus University/ University Hospital Dijkzigt, Rotterdam, The Netherlands.

SUMMARY

We have cloned and sequenced two homologous J regions of the T cell γ gene and localized one 5´ of each constant region. They have been numbered Jγ1.1 and Jγ2.1; other J regions have been renamed in a similar manner. Southern blot analysis suggests that there is comparatively more rearrangement to these J regions in thymocytes than peripheral blood T cells. We propose that either there is γ-chain gene rearrangement first to Jγ1.1 or 2.1 and then to Jγ1.3 or 2.3, or there is selected cell death of thymic T cells bearing a Jγ1.1 or 2.1 rearrangement.

INTRODUCTION

The T cell γ-chain gene encodes one subunit of a cell surface receptor found on a small subset of T cells (1,2). This receptor resembles the T cell antigen specific receptor found on most mature T cells (3). It is not necessary for cytotoxic or helper T cell function, and its precise role in the immune response remains obscure (4-7). To define the diversity and structure of the γ-chain, we and others have studied the γ gene locus. The γ gene undergoes somatic recombination in T cells and is structurally similar to the immunoglobulin and T cell receptor genes (3,8-11). There are only a few variable region genes encoded in the germline of mouse or man (9,12-17). However, the overall diversity of γ-chains is expanded by the addition of template-independent sequence (N segments) at the variable joining (VJ)[4] junction (5-7,9,12,14,17). Here we present evidence for greater germline J region diversity than previously suspected. We have determined the sequence and genomic location of two additional γ gene joining regions. Surprisingly, the proportion of rearranged γ genes that make use of these

* *Published in: J Immunol 1987;138:2687-90. The Vγ gene nomenclature is adapted to the nomenclature according to M.-P. LeFranc and T.H. Rabbitts (Cell 1986;45:237-46; Trends Biochem Sci 1989;14:214-8).*

joining regions is greater in thymocytes than in peripheral blood T cells.

MATERIALS AND METHODS

DNA preparation

Adult thymic tissue and leukemic blood were collected in accordance with accepted guidelines regarding patients' rights and approval; normal peripheral blood was obtained from volunteers. Lymphocyte populations of leukemic and normal peripheral blood were separated by fractionation on a Ficoll gradient (Lymphoprep, Bionetics), and the mononuclear cell fraction was collected for analysis. In all cases DNA was prepared by digestion with proteinase K in 0.5% sodium dodecyl sulfate (SDS), followed by extraction with phenol/chloroform and ethanol precipitation.

Cloning of joining regions

A rearranged fragment was cloned from DNA derived from a patient with T cell acute lymphoblastic leukemia ($CD4^-/CD8^+/CD6^-$). *Eco*RI digested DNA was size fractionated on a preparative agarose gel and was ligated into λgt 10. Recombinant phage were screened with a ^{32}P nick-translated variable region (Vγ II) probe derived from the previously described cDNA clone pTγ-10 (17). The 3´ portion of the cloned fragment λRBN was localized to the γ constant locus by hybridization to the previously described clone λ33, and comparison of restriction maps (13). A 250 basepair (bp) *Acc*I fragment containing the VJ junction (see Figure 2) was isolated from λRBN and was sequenced in both directions by using the dideoxy method of Sanger (18). This *Acc*I fragment when used as a probe in a Southern blot of cloned γ constant region DNA hybridized to a 1 kilobase (kb) *Eco*RI fragment of the previously described clone pγC1C2 (13). Nucleotide sequence analysis of this *Eco*RI fragment was carried out from an internal *Hin*dIII site and the 3´ *Eco*RI site.

Southern blotting

Genomic or cloned DNA was digested with *Eco*RI or *Pvu*II, was size fractionated on a 0.9% agarose gel, and was transferred to nitrocellulose. Hybridization was carried out with ^{32}P nick-translated probes as described (13). The Jγ1.1 probe is a 250 bp *Acc*I fragment that hybridizes to a 2.4-kb *Eco*RI genomic fragment containing Jγ1.1 and a 1-kb *Eco*RI genomic fragment containing Jγ2.1, and to rearranged fragments that make use of Jγ1.1 and Jγ2.1. This probe contains 50 bp of variable region sequence and produces a faint 5-kb band representing the germline Vγ II)(Vγ9) fragment. Rearranged bands noted in thymus DNA are not due to hybridization to variable region sequence, because a corresponding full length variable region probe fails to produce these bands. Also this pattern has subsequently been reproduced with a germline Jγ2.1 probe. The Jγ1.2 probe is a 2-kb *Hin*dIII fragment that hybridizes to a 6-kb germline *Pvu*II fragment and rearrangements to this J region. The Jγ1.3 probe is a 0.8-kb *Hin*dIII-*Eco*RI fragment that produces 1.2- and 3.5-kb *Eco*RI germline bands containing Jγ1.3 and Jγ2.3, respectively. Rearrangement patterns of leukemic T cells observed with this probe have been described (13).

RESULTS AND DISCUSSION

The human γ gene is encoded by two highly homologous constant regions and 11 variable regions constituting four V region families (10,11,14,17,19). Previous studies have identified a single joining region associated with each constant region (Jγ1 and Jγ2) that differ by only a single bp (17,19). Each of these J regions can be joined to one of the variable region genes.

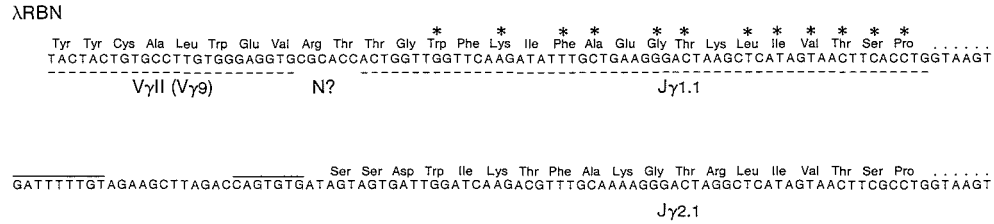


Figure 1. Nucleotide sequence of the productive rearrangement λRBN and germline Jγ2.1. Variable region and Jγ1.1 sequences of λRBN are underlined and labeled. Because of the loss of homology between λRBN and Jγ2.1 at the VJ junction, it is postulated that an N segment has replaced the 5′ Jγ1.1 sequence. Because the sequence of VγII (Vγ9) is derived from a cDNA clone (17), it is also possible that these bases represent the 3′ end of VγII (Vγ9). The characteristic heptamer and nonamer recombination signals of Jγ2.1 are overlined; RNA splicing signals are indicated by dots. Asterisks indicate conserved amino acids.

Most T cell leukemias contain rearranged γ-chain genes on both chromosomes in which a variable region gene has joined to one of these J segments (13). A third J region (JγP) was recently found in association with Cγ1 and was noted to rearrange to a γ V region (14).

Screening of T cell leukemias with a V region probe (VγII) has identified a rearranged fragment that does not hybridize to the known J regions. A 3.7-kb *Eco*RI fragment containing this rearrangement (λRBN) was cloned from the lymphoblasts of a patient (BN) with T cell acute lymphoblastic leukemia. Nucleotide sequence analysis at the VJ junction (Figure 1) revealed the VγII)(Vγ9) variable region joined to 60 bp of sequence with 50% homology to previously described J regions and was flanked at the 3′ end by GTAAGT, the same splice signal found in previously described γ J regions. This sequence thus represents a J segment located 5′ of Cγ1 (Figure 2). A region upstream of Cγ2 contains a highly homologous J region with charac-

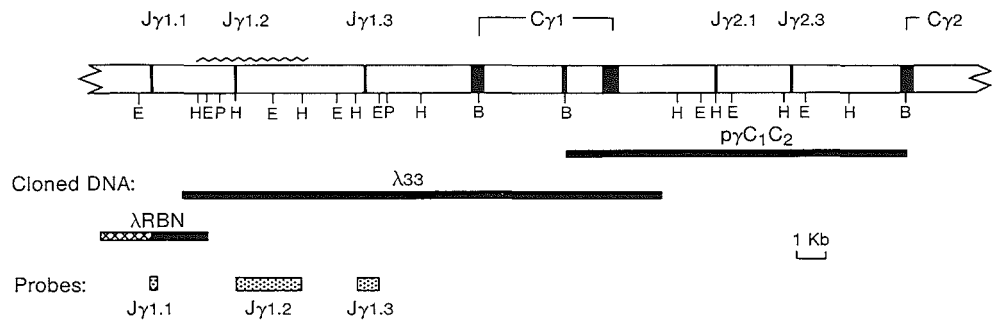


Figure 2. Genomic organization of joining and constant regions of the human γ-chain gene. J regions are indicated by vertical lines. J regions described here are Jγ1.1 and Jγ2.1. Previously described J regions have been renamed; Jγ1 was changed to Jγ1.3, Jγ2 was changed to Jγ2.3, and Jγp was changed to Jγ1.2. Constant region coding blocks are shown by hatching; genomic probes are stippled. Double cross-hatching of λRBN indicates variable region and 5′ untranslated V region sequence that has rearranged to Jγ1.1. The jagged line above Jγ1.2 and flanking sequence indicates a region that has no correlate upstream of Cγ2. Isolation and characterization of clones λ33 and pγC1C2 has been described (13). Only relevant restriction sites are shown. Abbreviations: E, *Eco*RI; H, *Hin*dIII; P, *Pvu*II; B, *Bam*HI.

teristic heptamer and nonamer 5′ sequences separated by 12 bp (Figure 1). The J segments described here are 70% homologous at the nucleotide level. Differences in the nucleotide sequence of these J regions at the 5′ end are probably due to an N region in the rearranged gene. By nucleotide analysis, the cloned rearrangement appears to encode a functional γ protein.

The genomic organization of the human γ constant and joining locus is shown in Figure 2. Because the J regions described here are the furthest 5′, they have been labeled Jγ1.1 and Jγ2.1; a tentative numbering scheme has been proposed for the other J regions. Jγ1.2 appears not to have a correlate associated with Cγ2. Probes derived from a 5-kb region flanking Jγ1.2 fail to hybridize to sequences between Cγ1 and Cγ2, indicating that this region of DNA was deleted at the time of duplication of the constant gene locus or was inserted after duplication.

The organization of the human and mouse γ genes now appear to be very different. The mouse gene has four constant regions, and each is associated with a single J region (7,9,15). Rather than the constant regions being linked, recent evidence closely links two of the mouse constant regions to variable regions (7,15). Other constant regions have a restricted pattern of rearrangement and may also be linked to variable regions. Although the organization of the γ locus was originally felt to be similar to the immunoglobulin λ locus, both the mouse and the human loci now appear to be quite different. The wide separation in the human of two or three J regions 5′ of the constant region (Jγ1.1 is 10 kb from Cγ1) is unique to this locus. The α-chain of the T cell receptor has J regions widely separated from the constant region, but appears to have a large total number of J regions (20). It is possible that other J regions may exist in the γ locus, but this could only be excluded by nucleotide sequence analysis of a large number of γ-chain cDNA. Additional J regions will be numbered in order of discovery by location and homology.

We have studied the use of γ J regions in different T cell populations by using J region probes in Southern blot analysis of adult thymocytes and peripheral blood T cells (Figure 1). Hybridization with the Jγ1.2 probe to thymus and peripheral blood T cell DNA did not reveal rearrangement in these T cell populations. However, rearrangement was found with the other J region probes under similar conditions, suggesting that Jγ1.2 is used less frequently in thymocytes and peripheral blood T cells than the other J regions. Jγ1.2 is capable of rearrangement, because it was initially characterized by cloning a genomic rearranged fragment from leukemic cells (14). Although the heptamer and nonamer recognition sequences associated with Jγ1.3 and Jγ2.3 are extremely similar to those found in Jγ2.1 (and presumably Jγ1.1), the heptamer and nonamer sequences of Jγ1.2 are quite different. Lefranc et al. (14) have suggested that such differences might lead to infrequent use.

Jγ1.3 and 2.3 are much more extensively used than Jγ1.2. Rearrangements to these J regions are easily visualized in both thymus and peripheral blood (Figure 3). This pattern is a composite of the rearrangements noted in a series of T cell leukemias (13), although the 9.5-kb band is not seen here and a new 2.2-kb band is faintly seen. Bands in the peripheral blood and thymus lanes appear to be comparable except for the 0.9-kb rearrangement. Thus all of the rearrangements but one are equally represented in thymus and peripheral blood T cell populations.

Probes containing Jγ1.1 or Jγ2.1 detect distinct rearranged bands when hybridized to thymus DNA, and there is diminution in intensity of the Jγ1.1 germline band (Figure 3). Although

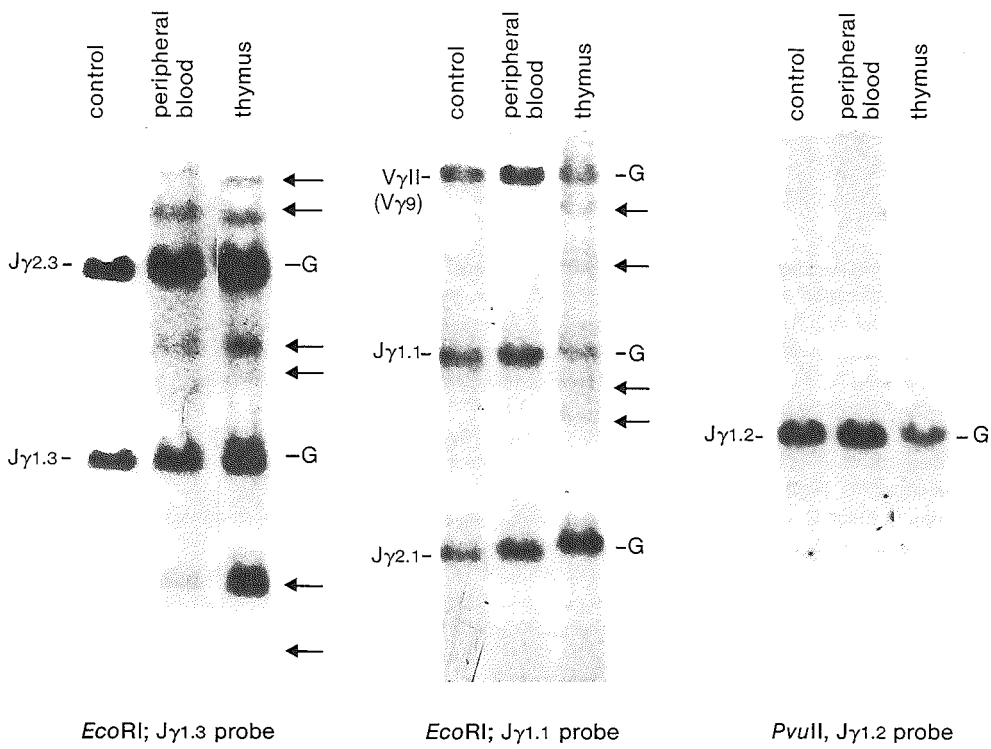


Figure 3. Southern blots using genomic J region probes. Probes were isolated as shown in Figure 2 and were explained in text. Germline fragments are indicated by arrowheads on the left of each blot and rearranged fragments are indicated by arrows on the right. Blots probed with Jγ1.3 and Jγ1.1 are *Eco*RI digests, and Jγ1.2 blots represent *Pvu*II digests. Jγ1.3 rearrangements are 5.3, 4.2, 2.5, 2.2, 0.9, and 0.58 kb. Jγ1.1 rearranged bands are 4.0, 3.3, 2.1, and 1.8 kb.

faint rearranged bands could be detected in peripheral blood on the original autoradiograph, rearrangement in thymocytes is more prominent than in peripheral blood T cells. This is in sharp contrast to the findings with the Jγ1.3 probe in which rearrangement is equally visible in both T cell populations. Thus the proportion of γ gene rearrangements that makes use of Jγ1.1 and Jγ2.1 is greater in thymocytes than peripheral blood T cells. Ten peripheral blood T cell samples and two thymus samples of comparable age have been examined with these probes with similar results (data not shown). The observed difference in degree of rearrangement to these J regions could represent a developmental pattern characterized by rearrangement of Jγ1.1/Jγ2.1 before rearrangement involving Jγ1.3 and Jγ2.3. Alternatively, T cells in which Jγ1.1 and Jγ2.1 are rearranged may be selected for cell death or retention in the thymus. The greater frequency of the 0.9 kb rearrangement detected in the thymus with the Jγ1.3 probe probably represents a similar phenomenon. Future studies should determine whether the γ genes rearranged in thymus produce functional γ-chains. Such studies will help to distinguish between these two models.

ACKNOWLEDGMENT. We thank Dr. Gary Tanigawa for valuable discussion and expert secretarial assistance.

This work was supported by National Institute for Health training (T. Quertermous, Grant T32-HL-07208; J.G. Seidman, Grant AI-19938 and AI-18436), the Netherlands Cancer Foundation (J.J.M. van Dongen) and the Mallinkrodt Foundation (J.G. Seidman).

REFERENCES

1. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
2. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 1986;322:179-81.
3. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell recognition. Annu Rev Immunol 1986;4:529-91.
4. Heilig JS, Glimcher LH, Kranz DM, Clayton LK, Greenstein JL, Saito H, Maxam AM, Burakoff SJ, Eisen HN, Tonegawa S. Expression of the T-cell specific γ gene is unnecessary in T cells recognizing class II MHC determinants. Nature 1985;317:68-70.
5. Rupp F, Frech G, Hengartner H, Zinkernagel RM, Joho R. No functional γ-chain transcripts detected in an alloreactive cytotoxic T-cell clone. Nature 1986;321:876-8.
6. Reilly EB, Kranz DM, Tonegawa S, Eisen HN. A functional γ gene formed from known γ gene segments is not neccessary for antigen-specific responses of murine cytotoxic T lymphocytes. Nature 1986;321:878-80.
7. Traunecker A, Oliveri F, Allen N, Karjalainen K. Normal T cell development is possible without "functional" γ chain genes. EMBO J 1986;5:1589-93.
8. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 1984;309:757-62.
9. Hayday AC, Saito H, Gillies SD, Kranz DM, Tanigawa G, Eisen HN, Tonegawa S. Structure, organization, and somatic rearrangement of T cell gamma genes. Cell 1985;40:259-69.
10. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T-cells and map to the short arm of chromosome 7. Nature 1985;316:549-52.
11. Lefranc MP, Rabbitts TH. Two tandemly organized human genes encoding the T-cell γ constant-region sequences show multiple rearrangement in different T-cell types. Nature 1985;316:464-6.
12. Kranz DM, Saito H, Heller M, Takagaki Y, Haas W, Eisen HN, Tonegawa S. Limited diversity of the rearranged T-cell γ gene. Nature 1985;313:752-5.
13. Quertermous T, Murre C, Dialynas DP, Duby AD, Strominger JL, Waldmann TA, Seidman JG. Human T-cell γ chain genes: organization, diversity, and rearrangment. Science 1986;231:252-5.
14. Lefranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T-cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
15. Iwamoto A, Rupp F, Ohashi PS, Walker CL, Pircher H, Joho R, Hengartner H, Mak T. T cell-specific γ genes in C57BL/10 mice. J Exp Med 1986;163:1203-12.
16. Garman RD, Doherty PJ, Raulet DH. Diversity rearrangement and expression of murine T cell gamma genes. Cell 1986;45:733-42.
17. Quertermous T, Strauss WM, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-7.
18. Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain terminating inhibitors. Proc Natl Acad Sci USA 1977;74:5463-7.
19. Lefranc MP, Forster A, Rabbitts TH. Rearrangement of two distinct T-cell γ-chain variable-region genes in human DNA. Nature 1986;319:420-2.
20. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.

CHAPTER 4.5

TWO TYPES OF GAMMA T CELL RECEPTORS EXPRESSED BY T CELL ACUTE LYMPHOBLASTIC LEUKEMIAS*

Jacques J.M. van Dongen[1], Ingrid L.M. Wolvers-Tettero[1], J.G. Seidman[2], Siew-Lan Ang[2], René J. van de Griend[3], Evert F.R. de Vries[5] and Jannie Borst[4]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands;
2. Department of Genetics, Harvard Medical School, Boston, MA 02115;
3. Department of Immunology, Radiobiological Institute TNO, Rijswijk, The Netherlands;
4. Department of Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands.

SUMMARY

$CD3^+$ cells, isolated from peripheral blood of two patients with T cell acute lymphoblastic leukemia (T-ALL) did not react with the monoclonal antibody WT31, which is thought to recognize a framework determinant on the conventional T cell receptor (TcR), consisting of disulfide-linked α and β chains. The T-ALL cells of neither patient synthesized TcR-α mRNA; the cells of patient DD contained only truncated (D-J) TcR-β mRNA, while the cells of patient HZ contained truncated as well as mature (V-D-J) TcR-β mRNA. The leukemic cells of both patients made TcR-γ mRNA. At the cell surface, the T-ALL cells of patient DD expressed a CD3-associated disulfide-linked dimer, which contained the TcR-γ protein. On the leukemic cells of patient HZ the TcR-γ protein was present as a 41-44 kDa CD3-associated subunit in a non-covalently linked form. The TcR-γ genes in the T-ALL cells of patient DD were rearranged exclusively to the Cγ1 locus, while in the T-ALL cells of patient HZ both Cγ1 alleles were deleted and rearrangement to the Cγ2 locus had occurred. The Cγ1 gene segment, just like the TcR-α and TcR-β gene segments contains a cysteine codon in its second exon. This cysteine residue is involved in the formation of the interchain disulfide bond. The human Cγ2 gene segment, however, does not contain a cysteine codon in its second exon. The absence of the cysteine residue in Cγ2 encoded TcR-γ chains explains the lack of an interchain disulfide bond in the TcR on the T-ALL cells of patient HZ. The TcR gene configuration as well as the expression of TcR mRNA and TcR protein as observed in these two leukemias, is consistent with a model for T cell differentiation in which the TcR-γ gene rearranges first to the Cγ1 locus prior to or coinciding with D-J joining of the TcR-β gene, followed by rearrangement to the Cγ2 locus and V-D-J joining of the TcR-β gene.

* *Published in: Eur J Immunol 1987;17:1719-28.*

INTRODUCTION

Many studies have indicated that the receptor by which mature T cells recognize antigen in combination with molecules of the major histocompatibility complex (MHC) is a disulfide-linked heterodimer, consisting of two structurally variable glycoproteins, the T cell receptor (TcR)-α and β chain (1,2). The TcR is closely associated with the CD3 antigen (3-7), which may play a role in signal transduction from the receptor to the cytoplasm (8-10). The TcR-α and TcR-β chains are encoded by variable, (diversity), joining and constant (V, D, J and C) gene segments which join through rearrangement during T cell differentiation in the thymus (11-15). Functionally rearranged TcR genes give rise to the expression of the TcR-CD3 protein complex on the cell membrane of mature T cells.

A third T cell specific rearranging gene, termed TcR-γ, has been identified (16-19). However, until recently the protein product of this gene remained elusive. The TcR-γ gene is transcribed abundantly early in thymic ontogeny, prior to TcR-α gene transcription (20,21). During further development TcR-γ gene transcription decreases, while TcR-α mRNA production increases (20,21). Mature T cells (T cell clones, blood T lymphocytes or mature T cell leukemias) may contain TcR-γ mRNA (15,22-28) which, however, is non-functional in the great majority of cases (26-31). These data suggested a role for the TcR-γ gene product during thymic differentiation (20,21).

Recently, the TcR-γ protein has been identified (32,33). In man, expression of the TcR-γ chain in association with CD3 has been demonstrated on T cell clones derived from normal peripheral blood (PB) (34-36), cord blood (37), fetal PB (38,39) and PB from immunodeficient patients (32,34,35) as well as on a thymocyte-derived T cell clone (33). Indirect evidence suggests that the percentage of TcR-γ-expressing $CD3^+$ T lymphocytes in adult PB is about 3%, while in the thymus this population is about 0.5% (40). In the mouse, it was shown that the TcR-γ protein is expressed on the cell surface of $CD3^+$ thymocytes at day 15 and 16 of fetal development (41,42) prior to expression of the TcR-αβ (42). After birth the TcR-γ chain is present on a small subset of murine thymocytes (42,43). The phenotype of TcR-γ-expressing cells is predominantly $CD3^+/CD4^-/CD8^-$ (32-36,40). Cells expressing TcR-γ protein may exert cytolytic activity (34,35,37). In this activity no involvement of MHC molecules as restriction elements has been demonstrated so far (34,35).

T cell acute lymphoblastic leukemias (T-ALL) represent the malignant counterparts of cells in various stages of T cell differentiation (44,45). Therefore, we recently analyzed a series of T-ALL to determine the configuration of the T cell receptor genes and their transcription as well as the expression of the TcR-CD3 complex (15). Abundant TcR-γ transcripts were detectable in the majority of thymocytic T-ALL, while the presumptive functional 1.3-kilobase (kb) TcR-β transcripts were present in almost all thymocytic T-ALL as well as mature T-ALL. TcR-α transcripts were only detected in mature $CD3^+$ T-ALL. However, in three $CD3^+$ T-ALL no TcR-α mRNA could be detected, indicating that they did not express the TcR-αβ. We suggest that such leukemias express an alternate TcR (15), since the expression of the CD3 antigen has been demonstrated to be dependent on the presence of a TcR (46-48). Here we describe the TcR-γ protein expression by two $CD3^+$/TcR-α $mRNA^-$ leukemias. Two different types of receptors were found in association with CD3: one leukemia expressed a disulfide-linked dimer involving the TcR-γ chain, while the other leukemia expressed a non-covalently linked TcR-γ

chain. Analysis of the configuration and expression of the TcR-γ genes enabled us to explain the occurrence of these two types of TcR-γ receptors.

MATERIALS AND METHODS

Cell samples

PB samples were derived from the T-ALL patient DD (11-year-old girl) and the T-ALL patient HZ (7-year-old girl). Mononuclear cells (MNC) were isolated from the PB samples by Ficoll-Paque (density: 1.077 g/mL; Pharmacia, Uppsala, Sweden). The T cell lines HPB-ALL and Jurkat were used as control sources for TcR-α, TcR-β and TcR-γ mRNA. DNA from B chronic lymphocytic leukemia (B-CLL) cells and DNA from normal PB cells was used as germline control in Southern blot analysis of the TcR genes. In some of the immunoprecipitation experiments TcR-αβ expressing leukemic T cells were used as a reference.

Immunologic marker analysis

The MNC from patient DD and patient HZ were analyzed for nuclear expression of terminal deoxynucleotidyl transferase (TdT), for cell membrane expression of the T cell markers CD1, CD2, CD3, CD4, CD5, CD6, CD7 and CD8, the B cell markers CD10, CD19 and CD20, the myeloid-monocytic markers CD14, CD15 and CD33, the HLA-DR antigen, the transferrin receptor and the interleukin 2 (IL2) receptor (CD25) as well as for reactivity with the monoclonal antibody (McAb) WT31, which recognizes a non-polymorphic epitope on TcR-αβ (49). General information about the antibodies used for the immunologic marker analysis is summarized in Table 1 (45). The indirect immunofluorescence stainings for TdT and the cell membrane markers were performed as described (45). The fluorescence staining was evaluated by use of Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, FRG).

Northern blot analysis

Total RNA was isolated using the LiCl/urea method (50) from frozen MNC of patient DD and patient HZ as well as from the T cell lines HPB-ALL and Jurkat. Approximately 15 μg of total RNA was size-fractionated on a 1.2% agarose gel (Sigma Chemical Co, St. Louis, MO) containing formaldehyde and blotted to Biodyne nylon membranes (Pall Ultrafine Filtration Corporation, Glen Core, NY). Specific RNA sequences were detected with ^{32}P-nick-translated TcR-α, TcR-β and TcR-γ cDNA probes (19,29,51,52). Hybridization and washing conditions were as described (50).

Southern blot analysis

DNA was prepared from frozen MNC of patient DD and patient HZ, as described (53). Fifteen μg of DNA was digested with *Eco*RI or *Bam*HI (New England Biolabs, Beverly, MA) and the fragments were size fractionated by electrophoresis through a 0.7% agarose gel. DNA was transferred onto Gene Screen Plus nylon membranes (New England Nuclear, Boston, MA) as described (50).

Rearrangements of the TcR-γ genes were detected by use of ^{32}P-nick-translated Jγ1.3 and Cγ probes (Figure 1) (18,19,29,54-56). The Jγ1.3 probe is a 0.8-kb *Eco*RI-*Hin*dIII fragment containing joining (J)γ1.3 segments; it hybridizes to both Jγ1.3 and Jγ2.3 gene segments (56). The Cγ probe is a 0.4-kb *Bam*HI-*Bam*HI fragment containing a part of the first exon of the constant (C)γ1 gene segments; it hybridizes to the first exon of Cγ1 and Cγ2 (18).

The configuration of the TcR-β genes was analyzed by use of ^{32}P-nick-translated Cβ and Jβ2 probes. The Cβ probe is a 0.7-kb *Pst*I-*Bgl*II fragment containing Cβ1 segments; it hybridizes to both Cβ1 and Cβ2 gene segments and allows detection of rearrangements in the TcR-β1 gene region in *Eco*RI digests (57). The Jβ2 probe consists

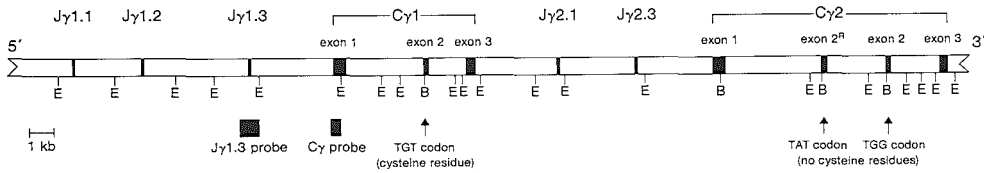


Figure 1. Organization of the J and C regions of the human TcR-γ gene (c.f. 54,56). The locations of the relevant *Eco*RI (E) and *Bam*HI (B) restriction enzyme sites are indicated. The solid bars represent the probes which were used for hybridization of the Southern blot filters. In the second exon of the Cγ1 genes the codon TGT codes for the cysteine residue which is presumably involved in the interchain disulfide bond, while in the duplicated second exon of the Cγ2 genes (exon 2^R and exon 2) this codon has been changed into TAT or TGG (29,54,55). Although in the majority of the Cγ2 gene-derived cDNA clones and genomic DNA clones a duplication of the second Cγ2 exon has been found, even a triplication of the second Cγ2 exon has been described in two cDNA clones derived from the TcR-γ protein-expressing T cell line PEER (55).

of contiguous 1.9-kb *Pvu*II-*Pvu*II and 1.5-kb *Pvu*II-*Eco*RV fragments containing Jβ2.3 through Jβ2.7; this probe does not hybridize to Jβ1 gene segments and was used to detect rearrangements in the TcR-β2 region in *Eco*RI digests (58). The absence or presence of TcR-β gene rearrangements was confirmed by *Bam*HI digests using the Cβ probe. Hybridization and washing conditions were as described (50).

Labeling

For cell surface radioiodination, 50 x 10^6-100 x 10^6 cells were labeled with $Na^{125}I$ (Amersham Corp., Buckinghampshire, UK), using 1,3,4,6-tetrachloro-3-α, 6-α-diphenylglycoluril (Iodogen, Pierce Chemical Co., Rockford, IL) as a catalyst.

Immunoprecipitation

Digitonin-mediated lysis was performed in immunoprecipitation buffer (IPB) consisting of 0.01 M triethanolamine-HCl, pH 7.8, 0.15 M NaCl, 1 mM phenylmethylsulfonylfluoride (PMSF), and 0.02 mg/mL ovomucoid trypsin inhibitor (TI) supplemented with 1% digitonin (Sigma). Lysates were pretreated by centrifugation and preclearing as described (34). Specific immunoprecipitation was carried out with the anti-CD3 McAb Leu-4 that had been coupled covalently to Sepharose beads by means of dimethylpimelimidate-HCl (Pierce Chemical Co.). Subsequently, beads were subjected to five washes in IPB with 1% digitonin.

To identify the TcR-γ protein the recently described rabbit antiserum was used, raised against a synthetic peptide of amino acids 117-136 of the TcR Cγ region (29,32). Prior to immunoprecipitation with this antiserum, lysates were denatured as described (32). Immunoprecipitates were recovered from the lysates by incubation with protein A-Sepharose beads and subjected to five washes as above. Reprecipitation of CD3 precipitates with the anti-Cγ serum was performed as described previously (34).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

Samples were analyzed on SDS-polyacrylamide (10-15%) gradient gels, using standard procedures, either under nonreducing conditions (1 mM iodoacetamide in SDS sample buffer), or under reducing conditions (5% (v/v) 2-mercaptoethanol in SDS sample buffer).

Two dimensional gel electrophoresis according to O'Farrell

Two-dimensional gel electrophoresis according to O'Farrell was performed using ampholytes (LKB, Bromma, Sweden) of isoelectric point (pI) 3.5-10, 4-6, 5-8 and 9-11 as 10:1:1:1 in the isoelectric focusing (IEF) dimension. The second dimension consisted of a 10-15% SDS-polyacrylamide gel.

Sialidase treatment

For sialidase treatment beads were resuspended in 50 μL of 0.05 M sodium acetate, pH 5.5, 0.9% NaCl, 0.1% $CaCl_2$, 1 mM PMSF, 0.02 mg/mL TI. Sialidase type VIII (Sigma) was added at 0.1 U/sample, and the mixture was incubated for 3 h at 37°C, while shaking.

RESULTS

Immunologic marker analysis of the leukemia cells of patient DD and patient HZ.

Immunologic marker analysis revealed that the majority of MNC isolated from PB of patients DD and HZ expressed the enzyme TdT and the transferrin receptor as well as the T cell markers CD2, CD3, CD5, CD6 and CD7 (Table 1). The cells did not express the B cell markers CD19 and CD20 and they were negative for the myeloid markers CD14, CD15 and CD33 as well as for the HLA-DR antigen and the IL2 receptor (CD25). This indicated that both patients had a T-ALL. Although both T-ALL were positive for CD3, they did not express the common determinant of the TcR-αβ, recognized by the McAb WT31 (49). The T-ALL cells of patient DD and patient HZ differed in expression of the common thymocytic marker CD1, the accessory molecules CD4 and CD8, and the common ALL antigen (CD10). The T-ALL cells of patient DD were clearly $CD1^-/CD4^-/ CD8^-/CD10^-$, while the majority of the T-ALL cells of patient HZ expressed the CD10 antigen and a part of the cells weakly expressed CD1, CD4 and CD8 (Table 1). These immunologic phenotypes would categorize the leukemia of patient DD as a "mature T-ALL" and the leukemia of patient HZ as a "$CD3^+$ common thymocytic T-ALL" (15).

TcR mRNA expression in the leukemic cells

Since the T-ALL cells seemed to lack TcR-αβ proteins at the cell surface while CD3 was expressed, it was analyzed which of the three known TcR genes were transcribed. Total RNA from the T cell lines HPB-ALL and Jurkat were used as controls (Figure 2). T-ALL cells of neither patient DD nor patient HZ synthesized TcR-α mRNA, while in HPB-ALL and Jurkat mature 1.7-kb TcR-α transcripts were present. In addition, immature 1.4-kb TcR-α mRNA was detected in HPB-ALL. In T-ALL cells of patient DD only truncated 1.0-kb TcR-β transcripts were found, while in T-ALL cells of patient HZ 1.0-kb transcripts as well as 1.3-kb TcR-β transcripts were found. Jurkat synthesized both 1.0 and 1.3-kb TcR-β mRNA, while in HPB-ALL only 1.3-kb mRNA was present. TcR-β transcripts of 1.0 kb are probably derived from incompletely rearranged TcR-β genes with D-J joining, while complete V-D-J joining results in 1.3-kb transcripts (2,15). TcR-γ mRNA was present in the cells of both patients as well as in the control

TABLE 1. Immunologic marker analysis of the T-ALL cells in the PB of patient DD and patient HZ.

Antibody	CD code[a] (antigen recognized)	% Positive MNC Patient	
		DD	HZ
Anti-TdT[b]	TdT	78	95
Leu-6[c]	CD1 (T6 antigen)	0	40[d]
Leu-5[c]	CD2 (T11 antigen)	95	98
Leu-4[c]	CD3 (T3 antigen)	84	94
Leu-3[c]	CD4 (T4 antigen)	1	59[d]
Leu-1[c]	CD5 (T1 antigen)	88	95
OKT17[e]	CD6 (Tp120 antigen)	69[d]	53[d]
3A1[f]	CD7 (Tp41 antigen)	96	98
Leu-2[c]	CD8 (T8 antigen)	0	14[d]
WT31[g]	TcR-$\alpha\beta$	0	2
VIL-A1[h]	CD10 (common ALL antigen)	0	73
B4[i]	CD19 (pan B cell antigen)	1	1
B1[i]	CD20 (B cell antigen)	1	1
My4[i]	CD14 (monocytic antigen)	0	1
VIM-D5[h]	CD15 (myeloid antigen)	2	2
My9[i]	CD33 (pan-myeloid antigen)	1	3
Anti-HLA-DR[c]	HLA-DR	18[d]	1
66IG10[j]	Transferrin receptor	70	87
2A3[c]	CD25 (IL2 receptor)	0	0

a. Cluster of differentiation (CD) as proposed by the Workshops on Human Leukocyte Differentiation Antigens (Paris, France, 1982; Boston, MA, 1984; Oxford, UK, 1986).
b. Supertechs, Bethesda, MD.
c. Becton Dickinson, Sunnyvale, CA.
d. Weak expression.
e. Ortho Diagnostic Systems, Raritan, NY.
f. American Type Culture Collection, Rockville, MD.
g. Dr. W. Tax, Nijmegen, The Netherlands.
h. Dr. W. Knapp, Vienna, Austria.
i. Coulter Clone, Hialeah, FL.
j. Dr. J.M. Van de Rijn, Amsterdam, The Netherlands.

HPB-ALL cells. Interestingly, TcR-γ transcripts in the T-ALL cells of patient DD were somewhat smaller (1.5-1.6 kb) than TcR-γ transcripts in the T-ALL cells of patient HZ and those of HPB-ALL (Figure 2c). TcR-γ mRNA could not be detected in Jurkat.

TcR-γ protein expression

Based on the lack of reactivity of the leukemic cells with the McAb WT31, the absence of TcR-α mRNA and the presence of TcR-γ mRNA, it was anticipated that these cells might express the protein product of the TcR-γ gene. The TcR-$\alpha\beta$ is expressed at the cell surface in obligatory association with the CD3 subunits (46-48). In analogy, it has been published that also the TcR-γ protein occurs in association with CD3 (32-36). Therefore, the T-ALL cells of patient DD and patient HZ as well as TcR-$\alpha\beta^+$ T-ALL cells as a control were radiolabeled at the cell surface with ^{125}I and lysed with a buffer containing the mild detergent digitonin, which preserves the noncovalent associations between the subunits of the TcR-CD3 complex. Subsequently, immunoprecipitation with the CD3 McAb Leu-4 was performed. Precipitates were

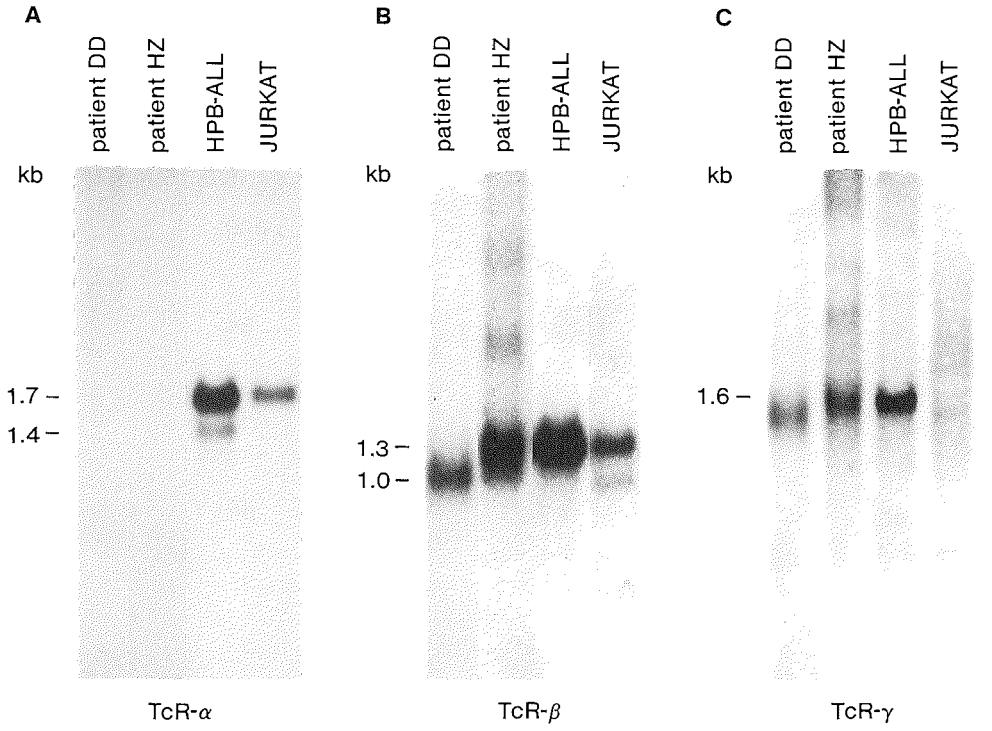


Figure 2. Northern blot analysis for the detection of transcripts of the TcR genes of the T-ALL cells of patient DD and patient HZ as well as of the T cell lines HPB-ALL and Jurkat. **A**: TcR-α transcripts; **B**: TcR-β transcripts; **C**: TcR-γ transcripts.

analyzed by SDS-PAGE under reducing as well as under nonreducing conditions.

In all precipitates both under reducing and nonreducing conditions the 19-29 kDa CD3 subunits could be detected. It was consistently observed that the CD3 components isolated from the T-ALL cells of patients DD and HZ displayed a lower molecular mass than the CD3 components isolated from the TcR-$\alpha\beta^+$ T-ALL cells. This difference appears to be due to differential glycosylation of the CD3 δ subunit (unpublished results). On the T-ALL cells of patient DD the CD3 components were associated with a heterogeneous band of 38-41 kDa under reducing conditions, which occurred as a disulfide-linked dimer of 70-75 kDa under nonreducing conditions (Figure 3a). For comparison, Figure 3b shows anti-CD3 precipitates from TcR-$\alpha\beta^+$ T-ALL cells. The TcR-α and β subunits of 44 and 37 kDa, respectively, were coprecipitated. These TcR chains formed an 80-kDa disulfide-linked dimer under nonreducing conditions (Figure 3b). On the T-ALL cells of patient HZ under both reducing and nonreducing conditions, CD3 was associated with a 41-44-kDa band, indicating that this component was not disulfide-linked to any other molecule (Figure 3b). In the immunoprecipitate from patient HZ we observed a weak band migrating at approximately 69 kDa under reducing conditions and at 60 kDa under nonreducing conditions. This band seemed to be nonspecific, since it was also present in the immunoprecipitate from the TcR-$\alpha\beta^+$ T-ALL cells under reducing conditions (Figure 3b).

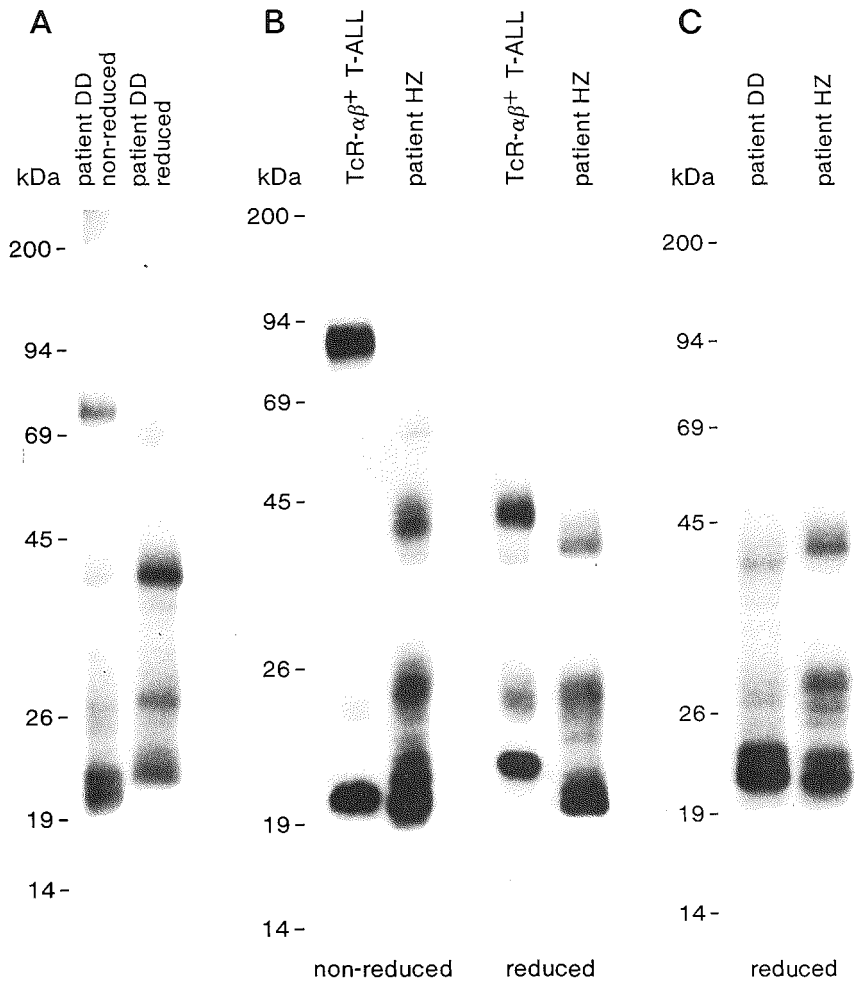


Figure 3. SDS-PAGE of CD3-associated components isolated from ^{125}I-labeled T-ALL cells of patient DD and patient HZ. **A**: Electrophoresis under nonreducing and reducing conditions of CD3 precipitates from T-ALL cells of patient DD. **B**: Electrophoresis under nonreducing and reducing conditions of CD3 precipitates from T-ALL cells of patient HZ compared with CD3 precipitates from TcR-$\alpha\beta^+$ T-ALL cells. **C**: Comparison of CD3 precipitates from the cells of patient DD and patient HZ under reducing conditions.

To determine whether the CD3-associated components contained the TcR-γ protein on the T-ALL cells of patients DD and HZ, a previously described rabbit anti-Cγ antiserum was used, which was raised against a synthetic peptide and is reactive with products of both Cγ1 and Cγ2 gene segments (29,32,34). The anti-Cγ serum precipitated bands with a mol. mass of 38-41 kDa and 41-44 kDa from lysates of the cells of patient DD and patient HZ, respectively, while no material was precipitated from the TcR$\alpha\beta^+$ T-ALL cells (Figure 4). By reprecipitation of CD3 precipitates with the anti-Cγ antiserum it was proven that the 38-41-kDa and 41-44-kDa components previously found to be associated with CD3 were at least in part identical to the

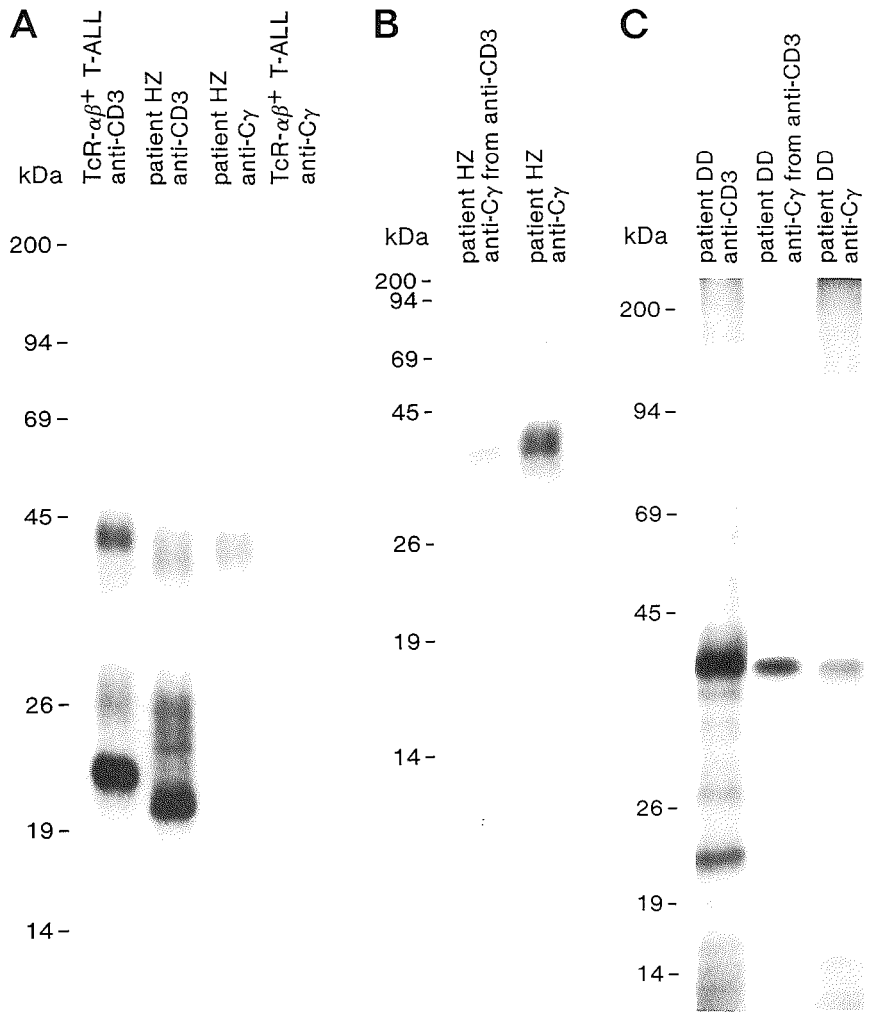


Figure 4. SDS-PAGE of CD3 and anti-Cγ immunoprecipitates from ^{125}I-labeled cells. **A**: Comparison of CD3 precipitates with anti-Cγ precipitates from the cells of patient HZ and the TcR-$\alpha\beta^+$ T-ALL cells. **B**: Reprecipitation of a CD3 precipitate from the T-ALL cells of patient HZ with the anti-Cγ antiserum, compared with a direct anti-Cγ precipitate. **C**: Reprecipitation of a CD3 precipitate from the T-ALL cells of patient DD with the anti-Cγ antiserum, compared with a CD3 precipitate and a direct anti-Cγ precipitate.

TcR-γ protein (Figure 4).

It was consistently found that the mol. mass of the TcR-γ protein expressed by the cells of patient DD was lower than that of the TcR-γ protein expressed by the cells of patient HZ (Figure 3c). This may be related to the smaller size of the TcR-γ transcripts in the cells from patient DD (Figure 2c), although such a discrepancy in mol. mass may also be caused by differential glycosylation.

Further characterization of the CD3-associated components

The immunoprecipitation experiments proved that the TcR-γ protein was expressed in noncovalent association with the CD3 subunits on the T-ALL cells of both patient DD and patient HZ. On the T-ALL cells of patient DD a disulfide-linked dimer was found, which could either be heterodimeric or homodimeric. This was not clear from SDS-PAGE, since the 38-41-kDa band was very heterogeneous. On the T-ALL cells of patient HZ the TcR-γ protein was not covalently associated. It was investigated by chemical cross-linking experiments, using three different reagents, whether the TcR-γ protein on T-ALL cells of patient HZ interacted noncovalently with another protein (heterodimer) or possibly with another TcR-γ subunit (homodimer). No evidence for such associations was found in repeated experiments, although cross-linking could be established between the CD3 components and the TcR chain (not shown).

To obtain additional information about the composition of the 21-44-kDa components on the T-ALL cells of patient HZ two-dimensional gel electrophoresis according to O'Farrell (IEF followed by SDS-PAGE) of anti-CD3 immunoprecipitates from digitonin-solubilized cells was performed. To eliminate charge heterogeneity due to the presence of sialic acids, one sample was also subjected to sialidase treatment. At the position of the 41-44-kDa components about six spots slightly differing in charge (pI 4-5) were present, which were reduced to three more basic spots (pI 5-6) upon sialidase treatment (Figure 5). The remaining heterogeneity may be due to incomplete sialidase digestion, since some charge heterogeneity was also remaining in the CD3 components. We suspect that the three remaining spots are representative of one TcR-γ chain, since no components with obviously different charge were detected. This suggests that the T-ALL cells of patient HZ express the TcR-γ protein in a monomeric form, although the presence of a second chain cannot be excluded.

Southern blot analysis

General remarks.

To investigate whether the absence or presence of interchain disulfide bonds in TcR-γ receptors is related to the TcR-γ gene configuration of the cells and whether the configuration of the TcR-β and TcR-γ genes is related to the differentiation stage of the cells, we performed Southern blot analysis.

Configuration of the TcR-γ and TcR-β genes in the T-ALL cells of patient DD.

To determine the configuration of the TcR-γ genes, *Eco*RI-digested DNA and *Bam*HI-digested DNA was analyzed by using the Jγ1.3 probe (see Figure 1 for the organization of the TcR-γ genes). In the *Eco*RI digest the germline band of 1.8 kb (Jγ1.3 gene segment) was absent, while the 3.3-kb germline band (Jγ2.3 gene segment) was present. In addition two non-germline bands of 2.5 and 2.3 kb were detected, which represent rearrangement type IV and VII, respectively, according to the classification of rearranged TcR-γ genes (15,19) (Figure 6a). These findings were confirmed in a *Bam*HI digest using the Jγ1.3 probe: the 23-kb germline band (Jγ1 gene segments) was absent, while two non-germline bands were detected

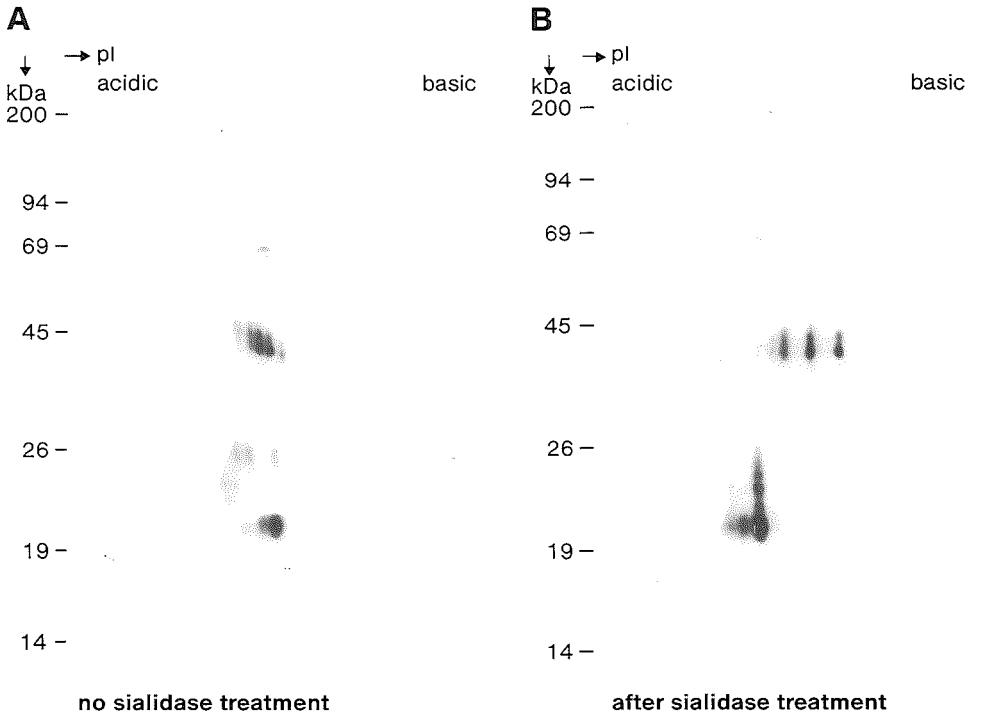


Figure 5. Two-dimensional gel electrophoresis according to O'Farrell (IEF followed by SDS-PAGE) of CD3 precipitates from ^{125}I-labeled cells of patient HZ. **A**: No sialidase treatment. **B**: After sialidase treatment.

in addition to the 12-kb germline band (Jγ2 gene segments, Figure 6c). In an *Eco*RI digest using the Cγ probe it was demonstrated that both the Cγ1 and Cγ2 gene segments were present (Figure 6b). These data indicate that both TcR-γ1 alleles were rearranged, while both TcR-γ2 alleles were in germline configuration.

The configuration of the TcR-β genes was determined by analysis of *Eco*RI-digested DNA by use of the Cβ probe and Jβ2 probe. In the *Eco*RI-digest using the Cβ probe one non-germline band was detected in addition to the 10.5-kb and 3.8-kb germline bands, while in *Eco*RI digests using the Jβ2 probe only the 4.1-kb germline band was detected. This indicates that one of the TcR-β1 alleles was rearranged, while the other TcR-β1 allele as well as both TcR-β2 alleles were in germline configuration (Table 2). Analysis of *Bam*HI digested DNA using the Cβ probe confirmed that indeed only one TcR-β allele was rearranged, while the other one was germline.

Configuration of the TcR-γ and TcR-β genes in the T-ALL cells of patient HZ.

Analysis of an *Eco*RI digest using the Jγ1.3 probe revealed that both germline bands were absent, while two non-germline bands could be detected: a 5.3-kb band and a 0.9-kb band, which represent rearrangement type II and type V, respectively (15,19) (Figure 6a). This was

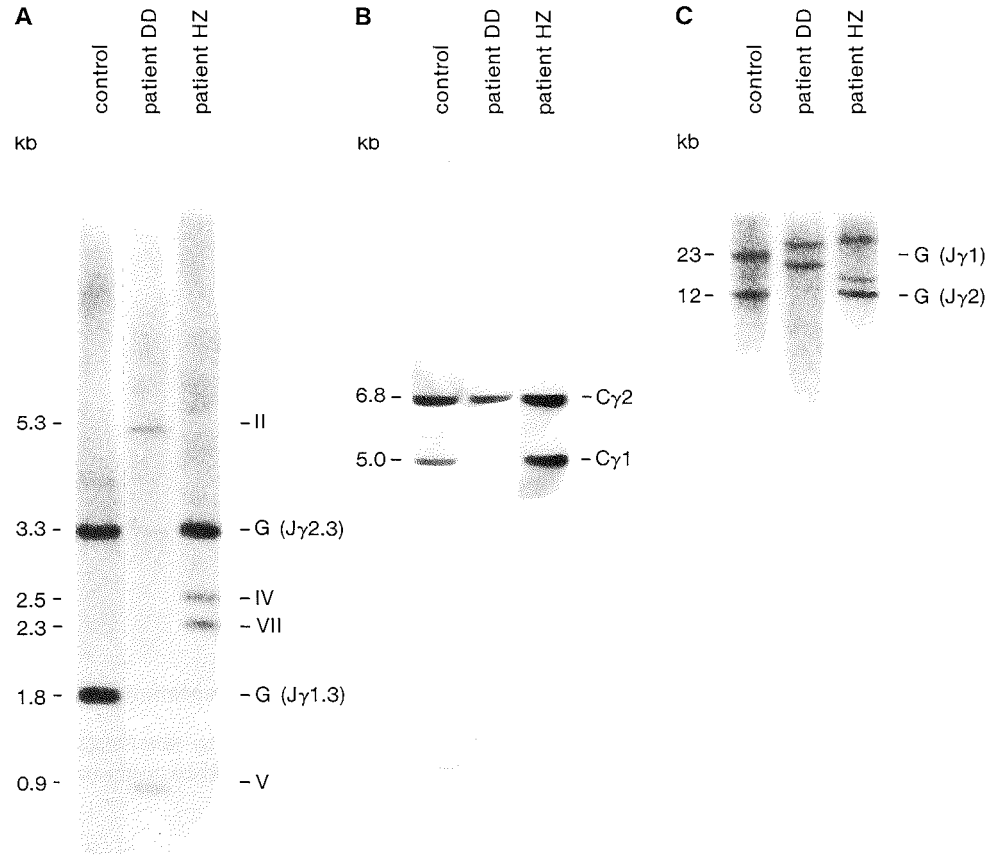


Figure 6. Southern blot analysis to determine the TcR-γ gene configuration of the T-ALL cells of patient DD and patient HZ. The size of the germline bands (G) is indicated. **A**: *Eco*RI-digested DNA from a control cell sample (B-CLL) and from the cells of patient DD and patient HZ. The filter was hybridized with the Jγ1.3 probe. The size of the non-germline bands type II, IV, V and VII (according to the classification of rearranged TcR-γ genes) are indicated. **B**: *Eco*RI-digested DNA from a control cell sample (normal PB) and from the cells of patient DD and patient HZ. The filter was hybridized with the Cγ probe. **C**: *Bam*HI-digested DNA from a control cell sample (B-CLL) and from the T-ALL cells of patient DD and patient HZ. The filter was hybridized with the Jγ1.3 probe.

confirmed in a *Bam*HI digest using the Jγ1.3 probe: both germline bands were absent, while two non-germline bands could be detected (Figure 6c). Analysis of an *Eco*RI digest using the Cγ probe revealed that both Cγ1 alleles were deleted, while Cγ2 gene segments were still present (Figure 6b). These data indicate that both TcR-γ1 alleles were deleted, while both TcR-γ2 alleles were rearranged.

Analysis of an *Eco*RI digest by use of the Cβ probe revealed that the 10.5-kb band was absent, while no non-germline bands could be detected. In an *Eco*RI digest using the Jβ2 probe the 4.1-kb germline band was absent, while two non-germline bands were seen. These data indicate that both TcR-β1 alleles were deleted, while both TcR-β2 alleles were rearranged.

TABLE 2. Southern blot analysis, Northern blot analysis and protein analysis of the T-ALL cells of patient DD and patient HZ.

	Patient	
	DD	HZ
Southern blot analysis[a]		
TcR-β1 genes	R/G	D/D
TcR-β2 genes	G/G	R/R
TcR-γ1 genes	R/R	D/D
TcR-γ2 genes	G/G	R/R
Northern blot analysis		
TcR-α mRNA	–	–
TcR-β mRNA	1.0 kb	1.0 and 1.3 kb
TcR-γ mRNA	1.5 – 1.6 kb	1.6 kb
Protein analysis		
Mol. mass of TcR-γ chain	38-41 kDa	41-44 kDa
Interchain disulfide bond	+	–

a. R, rearranged allele; D, deleted allele; G, allele in germline configuration.

Analysis of a *Bam*HI digest using the Cβ probe confirmed that both TcR-β alleles were rearranged (Table 2).

DISCUSSION

In a previous study we investigated TcR gene rearrangements and expression during T cell differentiation using T cell leukemias as a model system (15). In three out of twenty-two T-ALL the leukemic cells expressed CD3 on their cell surface without production of TcR-α mRNA, indicating that they lacked the TcR-αβ. We suggested that $CD3^+$/TcR-α $mRNA^-$ T cell leukemias may express an alternate TcR (15), since the cell surface expression of the CD3 antigen is dependent on the presence of a TcR (46-48). Therefore, we further analyzed two such leukemias, i.e. the T-ALL cells of patient DD and patient HZ.

Although the two T-ALL lacked TcR-α mRNA, they transcribed the TcR-γ genes as well as the TcR-β genes. Southern blot analysis of the TcR-γ genes of the T-ALL cells of patient DD showed that both TcR-γ1 alleles were rearranged, while both TcR-γ2 alleles were in germline configuration. Thus, it was concluded that the TcR-γ transcripts in these cells were derived from TcR-γ1 genes. In the T-ALL cells of patient HZ both TcR-γ1 alleles were deleted, while both TcR-γ2 alleles were rearranged, indicating that the TcR-γ transcripts in these cells were derived from TcR-γ2 genes. The T-ALL cells of patient DD and patient HZ also differed with respect to the TcR-β genes. The presence of the truncated 1.0-kb TcR-β transcripts in the T-ALL cells of patient DD indicated that the rearrangement of the TcR-β1 allele involved a D-J joining, while the presence of the 1.0-kb and 1.3-kb TcR-β transcripts in the T-ALL cells of patient HZ suggest that one of the rearranged TcR-β2 alleles had a D-J joining, while the other rearranged TcR-β2

TABLE 3. Data from the literature about 18 human TcR-γ chain expressing T cell populations.

Code	TcR-γ^+ T cells	Origin	TcR-β genes[a]	TcR-β mRNA[b] (kb)	TcR-γ genes	TcR-γ mRNA (kb)	Mol.mass TcR-γ chain (kDa)	Interchain disulfide bond (mol. mass of TcR in kDa)	Organization of the TcR	Ref.
A	T cell clone AK119	Pleural effusion	G	1,0?	R[a]	1.6	40 and 36	+ (70)	Homodimer	34
B	T cell clone AK615	Normal PB	G	[c]	R		40	+ (75)	Heterodimer	34
C	T cell clone AK925	PB from SCID patient[d]	G	1.0?[b]	R	1.6	40	+ (75)	Heterodimer	34
D-H	Five $CD3^+/CD8^-/CD4^-$ T cell lines[e]	Normal PB from five different donors	R	1.0	R	1.6	37 or 40	+ (90)	Heterodimer	36
I	T cell clone Wi.1	CSF from SSPE patient[f]	R[g]	1.0[g]	R[g]	1.6[g]	40 and 45	+ (85)	Homodimer?	61
J	T cell clone Mi.7	CSF from SSPE patient[f]	R[g]	1.0[g]	R[g]	1.6[g]	40 and 45	+ (85)	Homodimer?	61
K	T cell clone WM-14	Cord blood		1.0		1.6	43, 40, 38	+ (78)	Homodimer	37
L	T cell clone AK4	Normal PB	R	1.0	R	1.6	40	+ (75)	Heterodimer	34
M	T cell clone PBL-C1	Normal PB	R	1.0	R	1.6	40 and 36	+ (70)	Homodimer or Heterodimer	35
N	T cell clone F6C7	Fetal blood		1.0 and 1.3		1.6	44	+ (85)	Homodimer	38,39
O	T cell leukemia	PB from leukemia patient	G	–	R^h	1.6	43	–	Monomer?	60
P	T cell clone IDP2	PB from immuno-deficient patient	R	1.0	R	1.6	55 and 40	–	Heterodimer	32,35
Q	T cell clone CII	Thymus		1.0		1.6	44	–	Heterodimer	33
R	T cell line PEER	T cell leukemia		1.3		1.6	55	–	Monomer or Heterodimer	35,55,59

a. G; both alleles in germline configuration; R; at least one allele rearranged.
b. The TcR-β transcripts in the T cell clones AK119 and AK925 are derived from germline TcR-β genes. Such transcripts have been reported to be 1.1 kb (c.f. 14,15).
c. Blank space: not tested or not reported in the literature.
d. SCID, severe combined immunodeficiency.
e. Polyclonal T cell lines.
f. CSF, cerebrospinal fluid; SSPE, subacute sclerosing panencephalites.
g. Unpublished data (S.L. Ang).
h. In this leukemia the TcR-γ1 genes were deleted, while both TcR-γ2 genes were rearranged.

allele had a V-D-J joining (both TcR-β1 alleles were deleted in the latter T-ALL).

The T-ALL cells of patient DD as well as patient HZ expressed the TcR-γ protein at the cell surface in association with CD3. However, the configuration of the TcR of these leukemias was different. The T-ALL cells of patient DD expressed a disulfide-linked heterodimer or homodimer, while the T-ALL cells of patient HZ bore the TcR-γ protein in a non-disulfide-linked form. The DNA data revealed that the constant part of the TcR-γ protein is encoded by Cγ1 genes in the T-ALL cells of patient DD and by Cγ2 genes in T-ALL cells of patient HZ. In the second exon of the Cγ1 gene segment in human and mouse a cysteine residue is encoded (16,54,55). Such a cysteine codon is also found in the second exon of the C region gene segments of the TcR-α and β chains and is most likely responsible for the formation of the interchain disulfide bond (2). Genomic DNA clones and cDNA clones derived from human Cγ2-gene segments lack this cysteine codon (Figure 1) (29,54,55), which may well explain the absence of an interchain disulfide bond in the TcR-CD3 complex, expressed on the T-ALL cells of patient HZ.

In contrast to the Cγ1 genes, the second exon of the Cγ2 genes is duplicated, which will result in 48 extra exon basepairs in case of TcR-γ2 transcripts (29,54,55). This probably explains the slightly larger size of the TcR-γ transcripts in the T-ALL cells of patient HZ and the T cell line HPB-ALL, which both have deleted their TcR-γ1 gene alleles and use Cγ2 gene segments (Figure 2c). This may also explain the lower mol. mass of the TcR-γ1 chain (38-41 kDa) on the T-ALL cells of patient DD (Figure 3c), although such mol. mass differences can also be due to differences in glycosylation.

Disulfide-linked and non-disulfide-linked TcR-γ chains have been described previously (32-37,39,59-61). Data from the literature are summarized in Table 3. Initially, a TcR-γ^+ cell line derived from PB of an immunodeficient patient was described, which expressed the TcR-γ protein in non-covalent association with another protein, termed TcR-δ (32). Disulfide-linked dimers were subsequently found on T cell clones derived from a small subset of PB lymphocytes (34-39). The majority of these disulfide-linked dimers are heterodimeric (34,36), although a few TcR-γ homodimers have been described (34,37,39). While the T-ALL cells of patient DD express a disulfide-linked TcR-γ receptor, it is not clear what the exact organization of the receptor on the T-ALL cells of patient HZ is. Our data suggest that the latter T-ALL cells express a TcR-γ monomer, although it cannot be excluded that a partner of this chain, being either a TcR-γ or a TcR-δ chain, has remained undetected despite cross-linking experiments.

The T-ALL cells of patient DD and patient HZ have a thymocytic phenotype, since they both express the enzyme TdT in addition to T cell markers such as CD3 and CD5. Several studies have indicated that $CD3^+/TdT^+$ cells are not detectable outside the thymus (62,63). Based on the type of the expressed TcR-γ chain, their immunologic phenotype and the configuration of the TcR-γ and TcR-β genes, we tried to place the two T-ALL cells in a hypothetical differentiation scheme (Figure 7). This scheme is based on our own data and data from the literature (Tables 2 and 3). Most likely TcR-γ genes rearrange during differentiation in the thymus (15,20,21,64), although extrathymic TcR-γ gene rearrangement cannot be excluded, since functional TcR-γ transcripts have also been found in nude mice (65). In Figure 7 TcR-γ1 genes are depicted to rearrange prior to TcR-γ2 genes. Functional rearrangement of TcR-γ1 genes results in TcR-$\gamma1^+$ T cells, which still may have germline TcR-β genes (e.g. T cell clones AK119, AK615 and AK925), or rearranged TcR-β genes with D-J joining (the majority of TcR-$\gamma1^+$ T cells), or rearranged TcR-β genes with V-D-J joining (e.g. T cell clone F6C7) (c.f. Tables 2 en

3). If rearrangements of both TcR-γ1 alleles are nonfunctional, TcR-γ2 gene rearrangement occurs, deleting the TcR-γ1 gene segments. This gives rise to TcR-γ2$^+$ T cells, of which the majority will have rearranged TcR-β genes (e.g. T cell clones 1DP2 and CII, the T cell line PEER, and the T cell leukemia of patient HZ; c.f. Tables 2 and 3). If TcR-γ2 gene rearrangements are nonfunctional as well, the TcR-β and TcR-α gene rearrangements are completed, which may result in the different types of TcR-αβ$^+$ T lymphocytes (Figure 7).

The hypothetical differentiation scheme presented here also explains why virtually all T-ALL have undergone rearrangement of the TcR-β genes as well as TcR-γ genes (15,64,66,67). The majority of these leukemias probably belong to the TcR-αβ branches of the T cell lineage, while a minority belongs to the TcR-γ branches. The few CD3$^+$ T-ALL with germline TcR-β genes probably express TcR-γ chains, which has recently been proven for one of them (60,68). The precise frequency of TcR-γ$^+$/CD3$^+$ T-ALL is still unknown.

TcR-γ$^+$ T cells which migrate to the periphery may well constitute a functionally relevant lineage. T cell clones expressing either disulfide-linked or non-disulfide-linked TcR-γ receptors were found to exert broad cytolytic activity upon *in vitro* activation, in which involvement of MHC

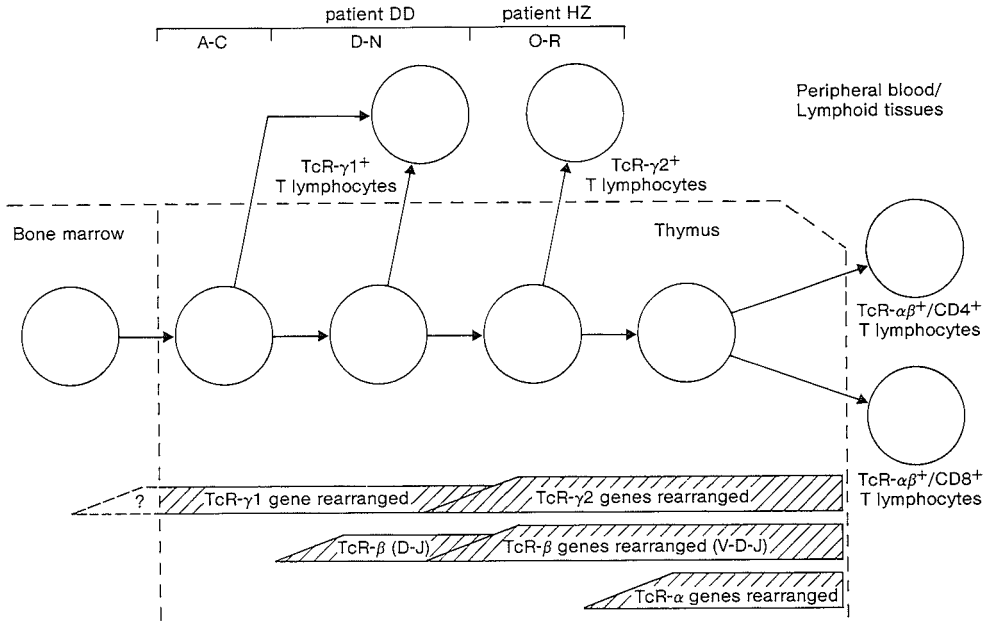


Figure 7. Hypothetical scheme of human T cell differentiation summarizing data about the TcR-γ$^+$ T cell leukemias presented here as well as data about TcR-γ$^+$ cells from the literature (2,15,32-39,42,59-61). The dotted lines represent the tissue compartments, where the cells in the various T cell differentiation stages are found. The horizontal bars summarize data concerning TcR gene rearrangement. Most likely TcR-γ genes rearrange during differentiation in the thymus, although extrathymic TcR-γ gene rearrangement cannot be excluded, since functional TcR-γ transcripts have also been found in nude mice (65). The letter codes above the scheme represent the various TcR-γ expressing T cell clones, T cell lines and T cell leukemias, which are located in this differentiation scheme according to their phenotypic and genotypic TcR characteristics (c.f. Tables 2 and 3). It is not yet clear whether TcR-γ1$^+$ cells and TcR-γ2$^+$ cells represent functionally different T cell subsets.

molecules as restriction elements on the target cells could not be detected (34-47). The T-ALL cells of the here presented patients, when tested within 24 h after thawing, did not exert MHC nonrestricted cytolysis (Dr. R.J. Van de Griend, unpublished results). It should be noted that such cells probably need to be activated or cloned to express this type of cytolytic activity, as was previously reported for $CD3^+/CD4^-/CD8^-$ cells derived from normal individuals (34,69). It was not possible, however, to expand *in vitro* the TcR-γ^+ leukemic cells of the two patients. Another explanation for the absence of lytic activity may be that these T-ALL cells were too immature to exhibit this function.

Future studies will have to elucidate the exact specificity and *in vivo* function of the TcR-γ^+ T cells. Furthermore, it is interesting that in PB of normal individuals only TcR-$\gamma1^+$ T cells have been found so far (34-39), while the reported TcR-$\gamma2^+$ cells are derived from thymus, thymic leukemias or PB of immunodeficient patients (32,33,59,60). Therefore, it should be studied whether TcR-γ^+ and TcR-$\gamma2^+$ cells represent functionally different T cell subsets.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof.Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Ms. W.M. Comans-Bitter for her skilful technical assistance, Dr. K. Hählen and Dr. G.E. van Zanen for providing the T-ALL cell samples, Mr. C. van Dijk (Audiovisueel Centrum of the Erasmus University) and Mr. T.M. van Os for their photographic assistance, Dr. A.D. Duby, Dr. C. Murre and Dr. T. Quertermous for kindly providing DNA probes, Dr. G. Peterman for assistance in preparation of the anti-Cγ antiserum and Ms. G. de Korte and Ms. J. de Goey-Van Dooren for typing this manuscript.

This work was supported by the Sophia Foundation for Medical Research (J.J.M. van Dongen and I.L.M. Wolvers-Tettero), the Netherlands Cancer Foundation (J. Borst and E.F.R. de Vries, grant N.K.I. 83-18) and National Institute for Health training (J.G. Seidman and S.-L. Ang, grants AI 18436 and AI 19148).

REFERENCES

1. Meuer SC, Acuto O, Hercend T, Schlossman SF, Reinherz EL. The human T-cell receptor. Annu Rev Immunol 1984;2:23-50.
2. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
3. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function. J Exp Med 1983;157:705-19.
4. Borst J, Alexander S, Elder J, Terhorst C. The T3 complex on human T lymphocytes involves four structurally distinct glycoproteins. J Biol Chem 1983;258:5135-41.
5. Oettgen HC, Kappler J, Tax WJM, Terhorst C. Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. J Biol Chem 1984;259:12039-48.
6. Brenner MB, Trowbridge IS, Strominger JL. Cross-linking of human T cell receptor proteins: Association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. Cell 1985;40:183-90.
7. Oettgen HC, Pettey CL, Maloy WL, Terhorst C. A T3-like protein complex associated with the antigen receptor on murine T cells. Nature 1986;320:272-5.
8. Samelson LE, Harford J. Schwartz RH, Klausner RD. A 20-kDa protein associated with the murine T-cell antigen receptor is phosphorylated in response to activation by antigen or concanavalin A. Proc Natl Acad Sci USA 1985;82:1969-73.
9. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, Stobo J. The role of the T3/antigen receptor complex in T-cell activation. Annu Rev Immunol 1986;4:593-619.
10. Cantrell DA, Davies AA, Crumpton MJ. Activators of protein kinase C down-regulate and phosphorylate the

T3/T-cell antigen receptor complex of human T lymphocytes. Proc Natl Acad Sci USA 1985;82:8158-62.
11. Samelson LE, Lindsten T, Fowlkes BJ, Van den Elsen P, Terhorst C, Davis MM, Germain RN, Schwartz RH. Nature 1985;315;765-8.
12. Collins MKL, Tanigawa G, Kissonerghis A-M, Ritter M, Price KM, Tonegawa S, Owen MJ. Regulation of T-cell receptor gene expression in human T-cell development. Proc Natl Acad Sci USA 1985;82:4503-7.
13. Royer HD, Ramarli D, Acuto O, Campen TJ, Reinherz EL. Genes encoding the T-cell receptor α and β subunits are transcribed in an ordered manner during intrathymic ontogeny. Proc Natl Acad Sci USA 1985;82:5510-4.
14. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46;75-87.
15. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
16. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 1984;309:757-62.
17. Hayday AC, Saito H, Gillies SD, Kranz DM, Tanigawa G, Eisen HN, Tonegawa S. Structure, organization, and somatic rearrangement of T cell gamma genes. Cell 1985;40:259-68.
18. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. Nature 1985;316:549-52.
19. Quertermous T, Murre C, Dialynas D, Duby AD, Strominger JL, Waldmann TA, Seidman JG. Human T-cell γ chain genes: organization, diversity, and rearrangement. Science 1986;231:252-5.
20. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.
21. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
22. Kranz DM, Saito H, Heller M, Takagaki Y, Haas W, Eisen HN, Tonegawa S. Limited diversity of the rearranged T-cell γ gene. Nature 1985;313:752-5.
23. Heilig JS, Glimcher LH, Kranz DM, Clayton LK, Greenstein JL, Saito H, Maxam AM, Burakoff SJ, Eisen HN, Tonegawa S. Expression of the T-cell-specific γ gene is unnecessary in T cells recognizing class II MHC determinants. Nature 1985;317:68-70.
24. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germline variable genes belonging to two subgroups. Cell 1986:45:237-46.
25. Zauderer M, Iwamoto A, Mak TW. γ gene rearrangement and expression in autoreactive helper T cells. J Exp Med 1986;163:1314-8.
26. Rupp F, Frech G, Hengartner H, Zinkernagel RM, Joho R. No functional γ-chain transcripts detected in an alloreactive cytotoxic T-cell clone. Nature 1986;321:876-8.
27. Reilly EB, Kranz DM, Tonegawa S, Eisen HN. A functional γ gene formed from known γ-gene segments is not necessary for antigen-specific responses of murine cytotoxic T lymphocytes. Nature 1986;321:878-80.
28. Iwamoto A, Rupp F, Ohashi PS, Walker CL, Pircher H, Joho R, Hengartner H, Mak TW. T cell-specific γ genes in C57BL/10 mice sequence and expression of new constant and variable region genes. J Exp Med 1986;163:1203-12.
29. Dialynas DP, Murre C, Quertermous T, Boss JM, Leiden JM, Seidman JG, Strominger JL. Cloning and sequence analysis of complementary DNA encoding an aberrantly rearranged human T-cell γ chain. Proc Natl Acad Sci USA 1986;83:2619-23.
30. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-7.
31. Yoshikai Y, Toyonaga B, Koga Y, Kimura N, Griesser H, Mak TW. Repertoire of the human T cell gamma genes: high frequency of nonfunctional transcripts in thymus and mature T cells. Eur J Immunol 1987;17:119-26.

32. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
33. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 1986;322:179-81.
34. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325:683-8.
35. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 1987;325:689-94.
36. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$, $CD8^-$, T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. J Exp Med 1987;165:1076-94.
37. Alarcon B, De Vries J, Pettey C, Boylston A, Yssel H, Terhorst C, Spits H. The T-cell receptor γ chain-CD3 complex: implication in the cytotoxic activity of a $CD3^+$ $CD4^-CD8^-$ human natural killer clone. Proc Natl Acad Sci USA 1987;84:3861-5.
38. Moingeon P, Ythier A, Goubin G, Faure F, Nowill A, Delmon L, Rainaud M, Forestier F, Daffos F, Bohuon C, Hercend T. A unique T-cell receptor complex expressed on human fetal lymphocytes displaying natural-killer-like activity. Nature 1986;323:638-40.
39. Moingeon P, Jitsukawa S, Faure F, Troalen F, Triebel F, Graziani M, Forestier F, Bellet D, Bohuon C, Hercend T. A γ-chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity. Nature 1987;325:723-6.
40. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
41. Nakanishi N, Maeda K, Ito K, Heller M, Tonegawa S. Tγ protein is expressed on murine fetal thymocytes as a disulphide-linked heterodimer. Nature 1987;325:720-3.
42. Pardoll DM, Fowlkes BJ, Bluestone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
43. Lew AM, Pardoll DM, Maloy WL, Fowlkes BJ, Kruisbeek A, Cheng S-F, Germain RN, Bluestone JA, Schwartz RH, Coligan JE. Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. Science 1986;234:1401-5.
44. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
45. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SC, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers 1987:87-116.
46. Weiss A, Stobo JD. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. J Exp Med 1984;160:1284-99.
47. Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, Calman AF, Terhorst C, Stobo JD, Weiss A. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. Nature 1985;316:606-9.
48. Van Dongen JJM, Geurts van Kessel HM, Wolvers-Tettero ILM, Versnel MA, Van Oudenaren A, Schoenmaker E, Hagemeijer A. Construction of human-mouse T cell hybrids that express human T cell-associated surface antigens and allow the chromosomal localization of these antigens. J Immunol 1986;137:1047-53.
49. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 1985;135:1922-8.
50. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1982:545.
51. Sim GK, Yagüe J, Nelson J, Marrack P, Palmer E, Augustin A, Kappler J. Primary structure of human T-cell receptor α-chain. Nature 1984;312:771-5.
52. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-6.
53. Jeffreys AJ, Flavell RA. A physical map of the DNA regions flanking the rabbit β-globin gene. Cell 1977;12:429-39.

54. Lefranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-9600.
55. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-8.
56. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
57. Morton CC, Duby AD, Eddy RL, Shows TB, Seidman JG. Genes for β chain of human T-cell antigen receptor map to regions of chromosomal rearrangement in T cells. Science 1985;228:582-5.
58. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.
59. Weiss A, Newton M, Crommie D. Expression of T3 in association with a molecule distinct from the T-cell antigen receptor heterodimer. Proc Natl Acad Sci USA 1986;83:6998-7002.
60. González-Sarmiento R, LeBien TW, Bradley JG, Greenberg JM, Seidman JG, Ang S, Kersey JH. Acute leukemia expressing the gamma gene product of the putative second T cell receptor. J Clin Invest 1987;79:1281-4.
61. Ang S-L, Seidman JG, Peterman GM, Duby AD, Benjamin D, Lee SJ, Hafler DA. Functional γ chain-associated T cell receptors on cerebrospinal fluid-derived natural killer-like T cell clones. J Exp Med 1987;165:1453-8.
62. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer M, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
63. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analyses: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986:113-33.
64. Davey MP, Bongiovanni KF, Kaulfersch W, Quertermous T, Seidman JG, Hershfield MS, Kurtzberg J, Haynes BF, Davis MM, Waldmann TA. Immunoglobulin and T-cell receptor gene rearrangement and expression in human lymphoid leukemia cells at different stages of maturation. Proc Natl Acad Sci USA 1986;83:8759-63.
65. Yoshikai Y, Reis MD, Mak TW. Athymic mice express a high level of functional γ-chain but greatly reduced levels of α- and β-chain T-cell receptor messages. Nature 1986;324:482-5.
66. Greenberg JM, Quertermous T, Seidman JG, Kersey JH. Human T cell γ-chain gene rearrangements in acute lymphoid and nonlymphoid leukemia: comparison with the T cell receptor β-chain gene. J Immunol 1986;137:2043-9.
67. Asou N, Matsuoka M, Hattori T, Kawano F, Maeda S, Shimada K, Takatsuki K. T cell γ gene rearrangements in hematologic neoplasms. Blood 1987;69:968-70.
68. Ogawa H, Sugiyama H, Oka Y, Maeda T, Komori T, Tani Y, Miyake S, Soma T, Maekura R, Yutani C, Masaoka T, Kishimoto S. Rearrangement of immunoglobulin heavy chain genes and T3 expression in the absence of rearrangement of T-cell receptor β-chain gene in a patient with T-cell malignant lymphoma. Leuk Res 1986;10:1369-75.
69. Van de Griend RJ, Tax WJM, Van Krimpen BA, Vreugdenhil RJ, Ronteltap CPM, Bolhuis RLH. Lysis of tumor cells by $CD3^+4^-8^-16^+$ T cell receptor $\alpha\beta^-$ clones, regulated via CD3 and CD16 activation sites, recombinant interleukin 2, and interferon β. J Immunol 1987;138:1627-33.

CHAPTER 4.6

DISTINCT MOLECULAR FORMS OF HUMAN T CELL RECEPTOR GAMMA-DELTA DETECTED ON VIABLE T CELLS BY A MONOCLONAL ANTIBODY*

Jannie Borst[1], Jacques J.M. van Dongen[2], Reinder L.H. Bolhuis[3], Peter J. Peters[4], David A. Hafler[5], Evert de Vries[1] and René J. van de Griend[3]

1. Department of Immunology, Netherlands Cancer Institute, Amsterdam;
2. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
3. Department of Immunology, Rotterdam Radiotherapeutic Institute, Rotterdam;
4. Department of Cell Biology, Faculty of Medicine, University of Utrecht, Utrecht, The Netherlands;
5. Center for Neurologic Diseases, Brigham and Woman's Hospital and Harvard Medical School, Boston, MA.

SUMMARY

A second type of T cell receptor (TcR) molecule has been identified on human and murine T lymphocytes, which involves the protein products of the γ and δ genes. T lymphocytes bearing this receptor may constitute a separate cell lineage with a distinct immune function. We have produced a monoclonal antibody (McAb), which specifically detects human T cell receptor (TcR)-$\gamma\delta$ in native as well as denatured states, this in contrast to previously used anti-γ chain peptide sera, which only reacted with denatured protein. The receptor occurs in different molecular forms, with or without interchain disulfide bonds, in which a δ chain may or may not be detected by cell surface iodination. The McAb is reactive with all these receptor forms. Therefore, this antibody could be used to determine the expression of TcR-$\gamma\delta$ on viable human T lymphocytes. In normal individuals, TcR-$\gamma\delta$ was found on a subset composing 2 to 7% of $CD3^+$ lymphocytes in peripheral blood and 0.1 to 1.0% in thymus. The majority of these cells do not express the CD4 or CD8 antigens, although a significant percentage of $CD8^+$ cells was found. TcR-$\gamma\delta^+$ cells in peripheral blood are resting lymphocytes, as judged by ultrastructural analysis. T cell clones with different receptor types can display major histocompatibility complex (MHC)-nonrestricted cytolytic activity, which is shown to be induced by the culture conditions, most likely by growth factors such as interleukin 2. This strongly suggests that TcR-$\gamma\delta$ does not play a role in target cell recognition in MHC-nonrestricted cytotoxicity. The anti-TCR-γ/δ-1 McAb can specifically induce cytotoxic activity in clones expressing the receptor, but in addition inhibit growth factor induced cytotoxicity, which indicates a regulatory role of the TcR-$\gamma\delta$/CD3 complex in MHC-nonrestricted cytotoxicity.

---

* *Published in: J Exp Med 1988;167:1625-44.*

INTRODUCTION

It is now clear that there are two types of T lymphocytes. Classical T cells recognize non-self antigens in the context of molecules of the major histocompatibility complex (MHC) by means of the T cell receptor (TcR), consisting of disulfide-linked α and β chains (1-3). A recently defined type of T cells, of which it is unclear what they recognize, uses a TcR encompassing the γ chain (4,5). All three chains, α, β, and γ, have a variable and a constant domain, encoded in different gene segments that join through rearrangement during T cell ontogeny (6-8). As compared with TcR-α and β-genes, the germline diversity of the TcR-γ gene is more limited (9). The human genome contains 11 variable, 5 joining, and 2 constant (C) gene segments in tandem (10-12). The γ gene is rearranged and expressed at an early stage in T cell development, coinciding with or preceding rearrangement and expression of respectively TcR-β and TcR-α genes (13-15). The TcR-γ chain forms either disulfide-linked or non-disulfide-linked dimers with the so-called δ protein, which is structurally distinct from γ (16). The gene encoding this protein is positioned in the TcR-α locus (17) and thus organized in a similar fashion as α, β, and γ genes, but its exact genomic organization has not yet been unravelled. Before cell surface expression, either type of TcR associates with the CD3 protein complex, consisting of four invariant subunits (18,19), which may play a role in signal transduction (20).

Upon in vitro activation, TcR-$\gamma\delta$-expressing cells can exert MHC-nonrestricted cytolytic activity towards allogeneic cells (21-23). In this and other functional aspects, TcR-$\gamma\delta^+$ cells share features with $CD3^-$ NK cells. On the other hand, they share features with $CD3^+$/TcR-$\alpha\beta^+$ MHC-restricted cells in the expression of CD3 and a potentially variable receptor.

The existence of the TcR-γ protein has first been demonstrated biochemically using anti-peptide sera, reactive with denatured protein. It was found in peripheral blood (PB) of normal individuals on cells derived from a small subset of $CD3^+/CD4^-/CD8^-$ cells (4,21,24) and on $CD3^+/CD4^-/CD8^-$ thymocytes in both mouse and man (5,25), which represent a small population in adults. Human TcR-$\gamma\delta$ has first been identified on $CD3^+$ lymphocytes, which failed to react with the McAb WT31 (26), thought to recognize a common determinant on TcR-$\alpha\beta$. Thus, TcR-$\gamma\delta^+$ cells may be identified using CD3 expression and lack of reactivity with WT31 McAb as criteria. However, it was important to make antibodies that positively identify TcR-$\gamma\delta$-expressing cells, particularly since we have recently found that TcR-$\gamma\delta^+$ cells may bind WT31 McAb under certain condition (27). We here report an McAb, which reacts with human TcR-$\gamma\delta$ in native as well as denatured states. This is in contrast to previously reported anti-peptide sera, which exclusively detected TcR-$\gamma\delta$ after denaturation. With this reagent one can assess TcR-$\gamma\delta$ expression in lymphoid tissues and blood, isolate TcR-$\gamma\delta$ expressing cells, manipulate their function and use it for isolation of the protein: studies needed to elucidate the role TcR-$\gamma\delta^+$ cells play in the immune system.

MATERIALS AND METHODS

Cells

PB samples were derived from healthy volunteers or from children hospitalized for afflictions other than infectious diseases or disorders of the immune system. Thymocyte samples were from children undergoing cardiac

surgery and from an 18-wk-old human fetus (abortion on medical indication). These human samples were obtained with permission of the Committee of Medical Ethics of the University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands. Mononuclear cells (MNC) were isolated from PB by density separation using Ficoll-Hypaque. Cloned lymphocytes were generated from PB of normal individuals in the case of AK4, AK781 (donor X; 22), 1012 (donor Y) and JS-93,-132,-228 (donor JS; 28), from pleural exudate of a patient with a mamma carcinoma in the case of AK119, from PB of a patient with T-γ lymphocytosis in the case of NK77 (29), and from PB of a patient with subacute sclerosing encephalitis in the case of Wi.K (30). Clones AK4, AK781 and AK119 were derived from PB-MNC directly by limiting dilution, clone 1012 was derived from $CD4^-/CD8^-$ sorted PB-MNC of donor Y, clone JS-228 was derived from $WT31^-$ sorted PB-MNC of donor JS. All clones were cultured in Yssel's medium (31), supplemented with 2% pooled human serum in round-bottom microwell plates (Greiner Labor Technik, Plidelsheim, FRG) in the presence of PHA, irradiated allogeneic PB-MNC and EBV-transformed B cells of the line JY (feeder cells), as described in detail elsewhere (32). The JY line (HLA-A2,2; B7,7; DR4,w6) was cultured in Yssel's medium supplemented with 2% pooled human serum. The human T leukemic cell line PEER (33), the Burkitt lymphoma cell line Daudi, the melanoma cell line MEWO, and the erythroid/myeloid cell line K562 were cultured in Iscove's medium containing 5% fetal calf serum under standard conditions.

Antibodies

The purified CD3 McAb WT32 (IgG2a) and McAb WT31 (IgG1) used for immunofluorescence, were kindly provided by Dr. W. Tax, Nijmegen, The Netherlands. Purified CD3, CD4, and CD8 McAb Leu-4, Leu-3a, and Leu-2a, respectively, as well as phycoerythrin-conjugates of Leu-4, Leu-3a, and Leu-2a, and goat anti-mouse Ig were from Becton Dickinson (San Jose, CA). For immunoprecipitation of CD3/TcR complexes the CD3 McAb 2G3 (IgG1) was used, which was a kind gift from Dr. Chang Geanwu, Dr. L. Willems Instituut, Diepenbeek, Belgium. Anti-Cγ antiserum was raised in rabbits against a synthetic peptide encompassing amino acids 137-157 of the human TcR-γ protein (4) and was kindly provided by Dr. J.G. Seidman, Harvard Medical School, Boston, MA.

Immunization protocol and generation of hybridomas

For immunization, CD3 immunoprecipitates were prepared as described below from a digitonin-solubilized mixture of cloned cells expressing disulfide-linked TcR-γδ. Antigen derived from 50×10^6 cells in the form of protein A-Sepharose/CD3 antigen complex was injected subcutaneously into BALB/c mice three times at 4-6-wk intervals. The fourth injection was done 7 wk after the third, directly into the spleen, using the same immunogen. The spleen was removed 4 days later. Spleen cells were fused to myeloma cells of the line SP2/0 at a ratio of 4:1 using polyethylene glycol. Hybridomas were seeded into 96-well microtiter plates (Costar, Cambridge, MA) at 10^5 spleen cell equivalents per well and grown and selected under standard conditions (hypoxanthin-aminopterin-thymidine selection).

Enzyme-linked immunosorbent assay (ELISA)

ELISA was used for screening of reactivity of hybridoma supernatants with intact cells: cells were fixed in microwells using poly-L-lysine and 0.025% glutaraldehyde. Specific binding of McAb to the cells was determined by incubation with biotinylated horse anti-mouse Ig, followed by incubation with biotinyl-peroxidase/avidin complex (Vector Inc., Burlingame, CA) and addition of 5-aminosalicylic acid as a substrate and H_2O_2 as a catalyst.

Immunofluorescence

Hybridoma supernatants were screened by indirect immunofluorescence using fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse F(ab)2 Ig (Tago Inc., Burlingame, CA) for staining and a fluorescence

microscope for evaluation. For identification and phenotyping of TcR-$\gamma\delta^+$ cells in PB and thymus of normal individuals described in Table 4, PB-MNC were first incubated with WT31 or anti-TCR-γ/δ-1 McAb followed by tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat anti-mouse Ig. Free binding sites of the second-step reagent were blocked with normal mouse serum and cells were incubated with FITC-conjugated McAb directed against CD3, CD4, or CD8 (Becton Dickinson). Samples were analyzed by fluorescence microscopy. For determination of the percentages $CD3^+$, $WT31^+$, and anti-TCR-γ/δ-1^+ cells, 1,000 cells were inspected for each sample. For determination of the CD4/CD8 phenotype, 100 anti-TCR-γ/δ-1^+ cells were inspected for each sample. As a control, normal mouse Ig was used as the first-step reagent. Other analyses were performed as indicated in the legends of the appropriate figures.

Cytotoxicity assays

Assays were carried out in serum-free medium. Cytotoxicity was measured in triplicate as percentage of ^{51}Cr-release in a 4-hour assay using round-bottom microtiter plates (Greiner Labor Technik). Effector cells were incubated with anti-TCR-γ/δ-1 McAb for 1 hour at 37°C before the assay at a dilution of ascitic fluid of 1:100 for induction or inhibition of cytotoxicity. The antibody remained present during the assay at a dilution of ascitic fluid of 1:200.

Radiolabeling

For cell surface radioiodination ~20×10^6 viable cells were washed with and resuspended in PBS, and labeled with 1-2 mCi $Na^{125}I$ (Amersham Co., Amersham, United Kingdom) using 1,3,4,6 tetrachloro-3α,6α-diphenyl glycoluril (Iodogen; Pierce Chemical Co., Rockford, IL) as a catalyst.

Immunoprecipitation

Cells were lysed in immunoprecipitation buffer (IPB), consisting of 0.01 M triethanolamine-HCl, pH 7.8, 0.15 M NaCl, 5 mM EDTA, 1 mM PMSF, 0.02 mg/ml ovomucoid trypsin inhibitor, 1 mM TLCK, 1 mM TPCK, 0.02 mg/ml leupeptin, supplemented with either 1% digitonin or 1% NP-40 as detergents. Nuclear debris was removed by centrifugation for 15 min at 13,000 g. Lysates were centrifuged for 30 min at 100,000 g and precleared by three subsequent incubations with 30 μl of a 10% v/v suspension of protein A-CL4B Sepharose beads (Pharmacia, Uppsala, Sweden) coated with normal mouse Ig. Specific immunoprecipitation was carried out for 2-4 hours with McAb, coupled covalently to protein A beads by means of dimethylpimelimidate-HCl (Pierce Chemical Co.). Beads were subsequently subjected to five washes in IPB with digitonin or NP-40 and samples were analyzed on 10-15% SDS-polyacrylamide gradient gels. Autoradiography was done using Kodak XAR-5 film in combination with intensifier screens (Cronex; Dupont Chemical Co., Newtown, CT).

Electron microscopy

PB-MNC were obtained from a normal donor. One-half of the cell sample was incubated with 0.01 mg/ml purified WT31 McAb and one-half with a 1:250 dilution of anti-TCR-γ/δ-1 ascitic fluid, followed by washing and incubation with purified rabbit anti-mouse Ig. Next, cells were washed and labeled with protein A-colloidal gold probes (9 nm). Gold particles were prepared by the tannic acid/citrate reduction procedure (34) and complexed to protein A (Pharmacia). All incubations were performed for 30 min at 4°C in the presence of sodium azide. Subsequently, cells were washed with ice-cold medium and fixed in a mixture of paraformaldehyde (2% w/v) and glutaraldehyde (2.5% v/v) in 0.1 M sodium cacodylate, pH 7.4, for 24 h at 4°C. Cells were pelleted in 3% agar (Agar Noble, Difco Laboratories, Inc., Detroit, MI) at 80°C. After postfixation in OsO_4(1% w/v) in cacodylate buffer for 2 hours, slices of agar were rinsed, dehydrated in graded ethanol, and embedded in Epon 812 using a LKB

Ultra Processor (LKB Produkter, Bromma, Sweden). Ultrathin sections (70 nm) were cut with an LKB-Nova ultramicrotome, contrasted with uranyl magnesium acetate and lead citrate staining and examined in a JEOL-1200 EX electron microscope (JEOL, Tokyo, Japan).

RESULTS

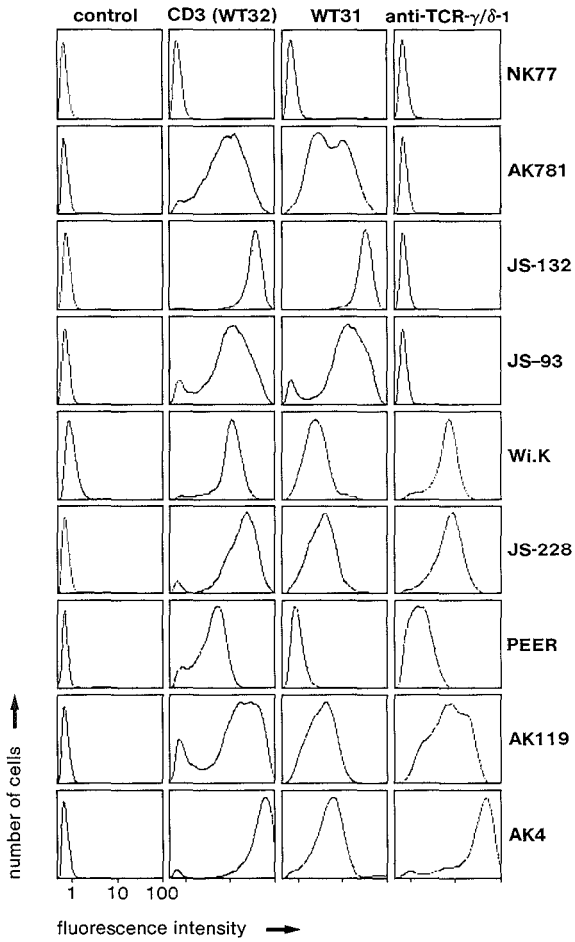
Generation of TcR-γδ-specific McAb

Human TcR-γδ proteins can be isolated in two ways. The intact CD3/TcR-γδ complex can be recovered with CD3 McAb from cells solubilized in the mild detergent digitonin (35), or from cells solubilized after chemical crosslinking of TcR and CD3 components (36). In addition, rabbit antisera have been described raised against synthetic peptides encompassing TcR-γ sequences (4,37). Such sera only detect TcR-γ protein after denaturation. To prepare McAb, which would detect TcR-γδ proteins in cell lysates as well as on intact, viable cells, we chose to immunize mice with CD3/TcR-γδ complexes isolated from digitonin-solubilized cells. A mixture of clones, expressing disulfide-linked dimers of TcR-γ and δ chains, was used.

In the relevant fusion, Ig was detected by ELISA in 344 hybridoma culture supernatants. These supernatants were assayed for reactivity in immunofluorescence with viable cells of clone 1012, which expresses disulfide-linked TcR-γδ. Only one supernatant contained reactivity with clone 1012, while no fluorescence was observed with the TcR-$\alpha\beta^+$ clone JS-132 or the EBV-transformed B cell line JY. This supernatant also specifically reacted with glutaraldehyde-fixed cells of clone 1012 in ELISA. This hybridoma was subcloned to give a stable clone, anti-TCR-γ/δ-1, which was used for further studies. The subclass of the McAb was IgG1.

Reactivity of anti-TCR-γ/δ-1 McAb with viable clones of various phenotypes

To assess the specificity of anti-TCR-γ/δ-1 McAb, first its reactivity was tested in immunofluorescence with PB-MNC of 18 normal donors. Positive PB-MNC ranged from undetectable to 5% as analyzed with a FACS. Such percentages of reactivity would be expected for a TcR-γδ-specific McAb, given previous results with WT31 McAb, and the suggestion that predominantly the small $CD3^+/CD4^-/CD8^-$ T cell subset would express TcR-γδ (38). Next, we determined reactivity in immunofluorescence of anti-TCR-γ/δ-1 McAb on a number of cloned cells of different phenotypes, representing different lymphocyte subsets and cells with different configurations of TcR-γδ: the NK clone NK77 ($CD3^-/CD4^-/CD8^-$), the nonlytic clone AK781 ($CD3^+/CD4^-/CD8^-$/TcR-$\alpha\beta^+$), the cytolytic clones JS-132 ($CD3^+/CD4^-/CD8^+$/TcR-$\alpha\beta^+$, MHC-class-I specific), and JS-93 ($CD3^+/CD4^+/CD8^-$/TcR-$\alpha\beta^+$, MHC-class-II specific), and the cytolytic clones Wi.K, JS-228, AK119, and AK4 ($CD3^+/CD4^-/CD8^-$, unknown specificity). It has been shown in immunoprecipitation experiments using anti-Cγ peptide serum that the latter four clones express the TcR-γ protein, be it in different configurations: non-disulfide-linked (Wi.K, JS-228) or disulfide-linked (AK119, AK4), and without (Wi.K, AK 119) or with (JS-228, AK4) direct evidence for participation of a δ chain in the receptor (see below). Also tested was the T leukemic cell line PEER, which was previously shown to express a non-disulfide-linked TcR-γ



	CD3 (WT32)	WT31	Anti-TcR-γ/δ-1
NK77	0	0	0
AK781	15	9	0.2
JS-132	49	42	0.2
JS-93	20	22	0.2
Wi.K	8.2	1.3	4
JS-228	11	1.5	3.5
PEER	4.5	0.3	1.1
AK119	31	4.4	14
AK4	59	7.8	43

Figure 1. Immunofluorescence stainings of various representative lymphocyte clones with anti-TCR-γ/δ-1 McAb. Cells were incubated with 0.01 mg/ml purified CD3 (WT32) or WT31 McAb, or with anti-TCR-γ/δ-1 McAb at 1:500 dilution of ascitic fluid, followed by incubation with FITC-conjugated goat anti-mouse F(ab)2 Ig. Fluorescence analyses were performed using a FACS IV (Becton Dickinson , San Jose, CA). The characteristics of the cell samples used are indicated in the text. Fluorescence intensity is plotted on a logarithmic scale. For exact comparison, fluorescence indexes, defined as [(fluorescence intensity sample)–(fluorescence intensity negative control)]/(fluorescence intensity negative control), are tabulated above.

chain of ~55 kDa (24,39), which is a different molecular (mol.) mass than found for the TcR-γ chains on Wi.K or JS-228 (see below).

As can be seen in Figure 1, neither the $CD3^-$ NK clone, nor any of the TcR-$\alpha\beta^+$ clones reacted with anti-TCR-γ/δ-1 McAb. In contrast, all TcR-$\gamma\delta$-expressing clones were positive. For comparison, fluorescence profiles obtained with CD3 and WT31 McAb are also shown. Fluorescence intensities are tabulated in the legend of Figure 1. Except NK77, all clones expressed CD3. TcR-$\alpha\beta^+$ clones reacted strongly with WT31 McAb, with intensities comparable

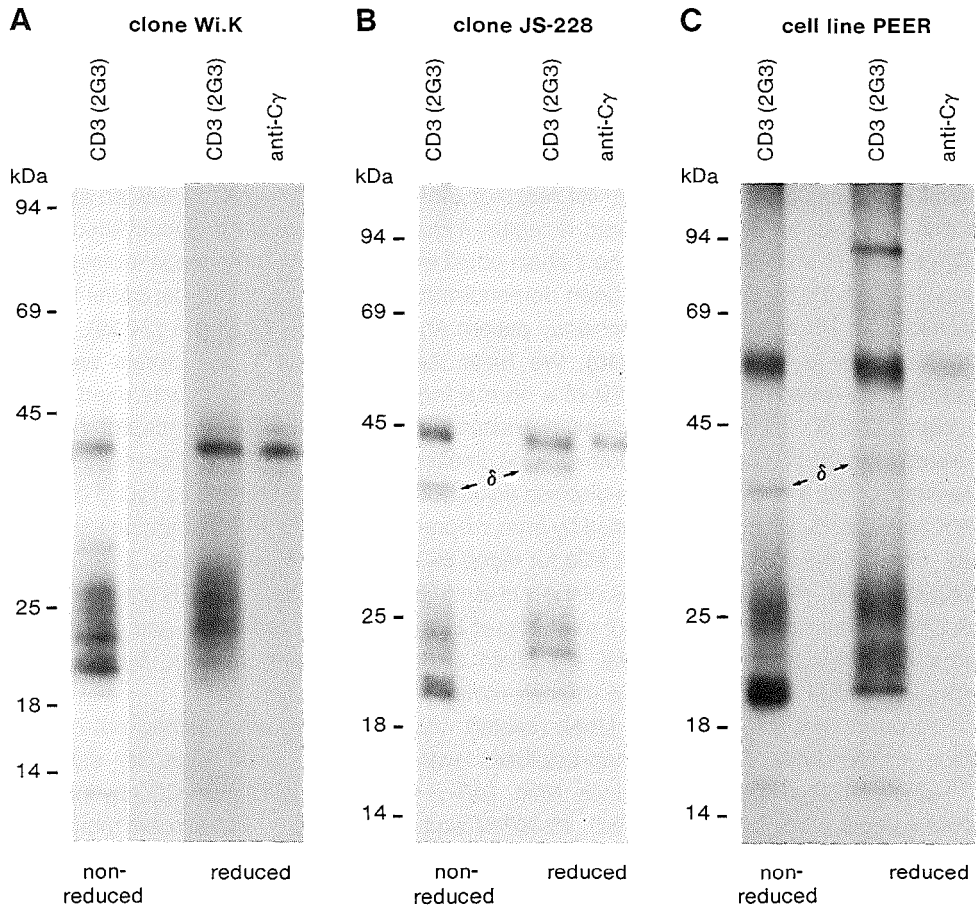


Figure 2. Characteristics of non-disulfide-linked TcR-γ(δ). Cells were labeled with ^{125}I, CD3 (2G3) immunoprecipitates were made from digitonin lysates and analyzed by SDS-PAGE under nonreducing or reducing conditions. For reprecipitation of TcR-γ chains, CD3 precipitates were incubated in IPB with 1% SDS and 2 mM DTT for 5 min at 68°C. The supernatant was diluted five times with IPB containing 1.5% NP-40, 20 mM iodoacetamide and 25 μg myoglobin, precleared once with normal mouse Ig-coated beads and subjected to immunoprecipitation with anti-Cγ antiserum. Arrows indicate the positions of the δ chain. **A**: CD3 precipitates (nonreducing and reducing conditions) and anti-Cγ precipitate (reducing conditions) from cells of clone Wi.K. **B**: CD3 precipitates (nonreducing and reducing conditions) and anti-Cγ precipitate (reducing conditions) from cells of clone JS-228. **C**: CD3 precipitate (nonreducing and reducing conditions) and anti-Cγ precipitate (reducing conditions) from cells of cell line PEER.

to those obtained with CD3 McAb. TcR-γδ$^+$ clones also bound WT31 McAb, but stained at low intensities, while on these clones fluorescence intensities with anti-TCR-γ/δ-1 McAb were more comparable to those obtained with CD3 McAb. These results clearly indicate that reactivity with anti-TcR-γ/δ-1 McAb, rather than lack of reactivity with WT31 McAb is a proper criterion for the identification of TcR-γδ-expressing cells.

Anti-TCR-γ/δ-1 McAb reacts with different molecular species of TcR-$\gamma\delta$ in immunoprecipitation

In contrast to TcR-$\alpha\beta$, TcR-$\gamma\delta$ occurs in various molecular configurations. We have previously described (21) cytolytic clones such as AK4, expressing a TcR consisting of a 40-kDa γ chain disulfide linked to a 43-kDa δ chain. Clone 1012 and eight other clones derived from the same culture express a similar receptor (not shown). Clone AK119 was an exception, in that it expressed a disulfide-linked receptor, including a differentially glycosylated γ chain migrating at 36 and 40 kDa, while no δ chain could be detected by cell surface iodination (21). Non-disulfide-linked TcR-$\gamma\delta$ have been demonstrated on a growth factor-dependent cell line derived from PB of an immunodeficiency patient (4), a leukemic T cell line (24,39), and PB-MNC from a leukemic patient (40). We have derived several T cell clones expressing non-disulfide-linked TcR-$\gamma\delta$ from PB of a normal individual, one of which was used in these studies (JS-228). In addition, a clone expressing a non-disulfide-linked receptor has been isolated from PB of a patient with autoimmune disease (clone Wi.K). Figure 2 demonstrates the presence of the γ chain in the non-disulfide-linked CD3-associated receptors found on clones Wi.K and JS-228, which was identified by immunoprecipitation with anti-Cγ peptide serum. The mol. mass of the γ chain was ~41 kDa for clone Wi.K and ~44 kDa for clone JS-228. This in contrast to the ~55 kDa mol. mass of the γ chain found on the cell line PEER (24,39). In clone JS-228 a δ chain could be detected, migrating at 37 kDa under nonreducing and 40 kDa under reducing conditions. In clone Wi.K a δ chain could not be demonstrated by cell surface iodination. Table 1 summarizes the different molecular forms of TcR-$\gamma(\delta)$.

To prove that anti-TCR-γ/δ-1 McAb reacted with TcR-$\gamma\delta$, we have carried out reprecipitation experiments. CD3 immunoprecipitates were prepared from digitonin solubilized, ^{125}I-labeled TcR-$\gamma\delta$-expressing clones. Next, TcR-$\gamma\delta$ was dissociated from CD3 by incubation of the immunoprecipitate in buffer with 1% NP-40 and 1% SDS, for 30 min at room temperature. The solubilized material was subjected to immunoprecipitation with anti-TCR-γ/δ-1 as detailed in the legend of Figure 3. In Figure 3 the CD3/TcR-$\gamma(\delta)$ complexes as isolated by CD3 McAb are shown, as well as TcR-$\gamma(\delta)$ reprecipitated with anti-TCR-γ/δ-1 McAb. The antibody could be used to reprecipitate both types of disulfide-linked receptors from TcR-$\gamma(\delta)^+$ clones 1012

TABLE 1. Molecular forms of TcR-$\gamma(\delta)$.

Clone	Receptor type	Interchain S-S bond	Mol. mass of γ chain in kDa	Mol. mass of δ chain in kDa
AK119	γ (δ)	+	~40+~36	ND[a]
AK4	γ δ	+	~40(+~36)[b]	~43
1012	γ δ	+	~40(+~36)	~43
Wi.K	γ (δ)	–	~41	ND
JS-228	γ δ	–	~44	~40 (R),[c] ~37 (NR)[c]
PEER	γ δ	–	~55	~40 (R), ~37 (NR)

a. ND, not detected.
b. Occasionally detected.
c. R, reducing conditions; NR, nonreducing conditions.

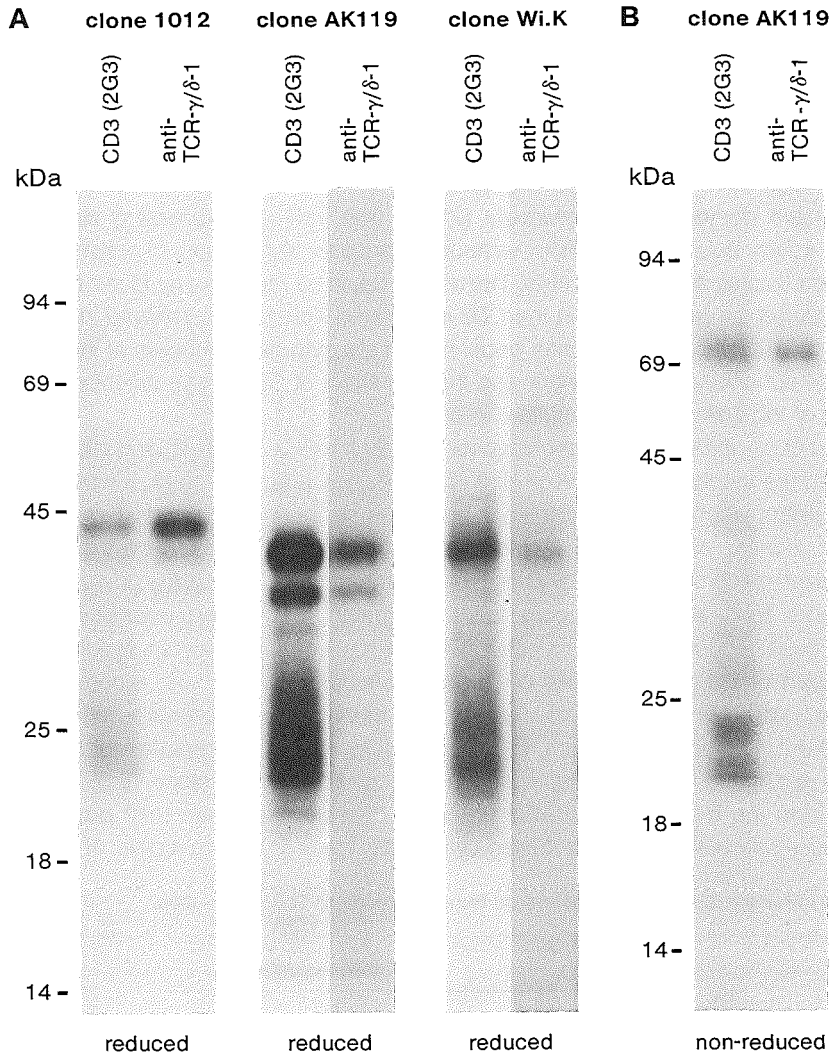


Figure 3. Biochemical proof that anti-TCR-γ/δ-1 McAb detects TcR-γ(δ). CD3 precipitates were prepared as described for Figure 2. TcR-γ(δ) was dissociated from CD3 by incubation of one-half of the CD3 precipitates in IPB with 1% NP-40 and 1% SDS for 30 min at room temperature. The supernatant was diluted five times with IPB containing 1.5% NP-40 and 25 μg myoglobin, precleared once with normal mouse Ig-coated beads, and subjected to immunoprecipitation with anti-TCR-γ/δ-1 McAb and analyzed by SDS-PAGE under nonreducing or reducing conditions. **A**: CD3 and anti-TcR-γ/δ-1 precipitates from cells of clone 1012, clone AK119 and clone Wi.K under reducing conditions. **B**: CD3 and anti-TcR-γ/δ-1 precipitates from cells of clone AK119 under nonreducing conditions.

(Figure 3A) and AK119 (Figure 3A and 3B), as well as the non-disulfide-linked receptors from TcR-γ(δ)$^+$ clones Wi.K (Figure 3A) and JS-228 (not shown).

Thus, anti-TCR-γ/δ-1 McAb reacts with all different types of TcR-γ(δ) described thus far, in

native state as present on intact viable cells, as well as in denatured state after detergent solubilization.

Anti-TCR-γ/δ-1 McAb affects the function of cytolytic clones

We have demonstrated previously (21,22) that TcR-$\gamma(\delta)$-expressing clones can exert cytolytic activity upon in vitro culture, without evidence for involvement of polymorphic domains of MHC molecules on the target cells. The activity could be regulated by McAb directed at CD3, which opened the possibility that the CD3-associated TcR-$\gamma\delta$ was involved in the recognition of these target cells. Another option would be that the cytotoxic spectrum of the clones did not reflect the specificity of the receptor and that the effect of CD3 McAb reflected the regulatory capacity of the TcR-$\gamma\delta$/CD3 complex.

It had been observed that lymphokines, such as interleukin 2 (IL-2) and interferon β (IFN-β) enhanced the cytotoxicity of TcR-$\gamma\delta$-expressing clones (22). Therefore, it was investigated in the first place, whether the lymphokines produced by the feeder cell mixture used to culture TcR-$\gamma\delta^+$ clones, affected the cytotoxic potential of the clones. For this purpose, two TcR-$\gamma\delta$-expressing clones, 1012 and Wi.K, were cultured for 5 days in the presence of irradiated feeder cells. Next, when feeder cells had disintegrated, clones were washed and cultured for an additional 72 hours in Yssel's medium with 2% pooled human serum, lacking lymphokines. To one part of the cells, deprived of feeder cell factors for 48 hours, recombinant IL-2 was added at 1,000 U/ml for 24 hours. Control cells of the same batch were kept in culture for 8 days without changing the medium. Then, cytotoxicity was tested towards four allogeneic tumor cell lines of different histologic origin. In Table 2 it can be seen that clone 1012, when harvested directly from the 8-day culture in the presence of feeder cells and their products (control), displayed significant MHC-nonrestricted cytotoxicity towards all target cells. The cytotoxicity of

TABLE 2. Influence of feeder cell factors on the cytotoxicity of TcR-$\gamma\delta^+$ clones.

Clone		Percent cytotoxicity							
		K562[a]		JY		Daudi		MEWO	
		10:1[b]	2:1	10:1	2:1	10:1	2:1	10:1	2:1
1012:	Control[c]	52	32	35	26	69	67	39	22
	– factors[d]	8	0	19	10	73	37	11	2
	+ IL-2[e]	66	46	55	44	71	73	53	31
Wi.K:	Control	32	9	8	3	4	2	13	3
	– factors	7	0	11	3	0	0	1	0
	+ IL-2	31	18	13	14	0	0	9	4

a. Target cells used.
b. Effector cell/target cell ratio.
c. Control: culture with feeder cells for 8 days.
d. – factors: culture as in "control", but harvested and washed on day five subsequently cultured in absence of lymphokines for 72 hours.
e. + IL-2: culture as in "– factors", but cultured with 1,000 U/ml IL-2 during the last 24 hours.

factor-deprived cells of clone 1012 towards three of four target cells was drastically reduced, while it could be enhanced to a level comparable to or higher than that of control cells by the addition of IL-2. Clone Wi.K displayed significant cytotoxicity only towards K562 cells, where similar effects of factor depletion and IL-2 addition were observed as for clone 1012. IL-2 could not induce significant cytotoxicity of Wi.K towards the other three target cell lines.

Secondly, it was determined whether anti-TCR-γ/δ-1 McAb affected the function of the clones. Clones 1012, Wi.K, and as a control, the TcR-$\alpha\beta^+$ cytotoxic clone JS-132, specific for a determinant on the HLA-A2 molecule, were used. To discriminate between factor-induced cytotoxicity and possible cytotoxicity reflecting the specificity of the TcR molecules, the assay was carried out with clones kept in culture with feeder cells for 8 days (control), as well as with clones harvested and washed on day six and cultured in the absence of lymphokines as described above, for 48 hours. The results of this experiment are shown in Table 3. Clone JS-132 displayed nonspecific cytotoxicity towards K562 and Daudi, which was strongly reduced in the absence of factors, while its specific activity towards JY and MEWO involving recognition of HLA-A2 by the TcR was retained. Again, the effect of factors produced by the feeder cell mixture on the cytotoxicity of clone 1012 was evident. In this experiment also the cytotoxicity of clone 1012 towards the cell line Daudi was reduced in the absence of factors, in contrast to the results shown in Table 2. Clone Wi.K did not display any cytotoxicity in this experiment. However, anti-TCR-γ/δ-1 McAb induced significant cytotoxicity of clone Wi.K towards K562 and Daudi, but not to JY or MEWO. The McAb had no effect on the cytotoxicity of clone 1012 to-

TABLE 3. Influence of anti-TCR-γ/δ-1 McAb on the cytotoxicity of TcR-$\gamma\delta^+$ clones.

Clone		Percent cytotoxicity							
		K562[a]		JY		Daudi		MEWO	
		10:1[b]	2:1	10:1	2:1	10:1	2:1	10:1	2:1
1012:	Control[c]	37	11	23	10	55	21	42	17
	Control + McAb	28	18	7	4	13	5	7	2
	– factors[d]	0	0	10	5	26	7	11	4
	– factors + McAb	10	6	1	1	3	2	1	1
Wi.K:	Control	3	0	0	0	2	0	3	1
	Control + McAb	57	46	4	2	63	33	13	6
	– factors	0	0	0	0	2	1	1	0
	– factors + McAb	30	25	0	0	28	13	2	1
JS-132:	Control	55	32	88	84	26	9	64	43
	Control + McAb	61	38	81	84	36	9	52	47
	– factors	15	6	90	86	2	1	54	43
	– factors + McAb	20	10	86	81	3	1	48	40

a. Target cells used.
b. Effector cell/target cell ratio.
c. Control: culture with feeder cells for 8 days.
d. Factors: clones harvested and washed on day six and cultured in absence of lymphokines for 48 hours.

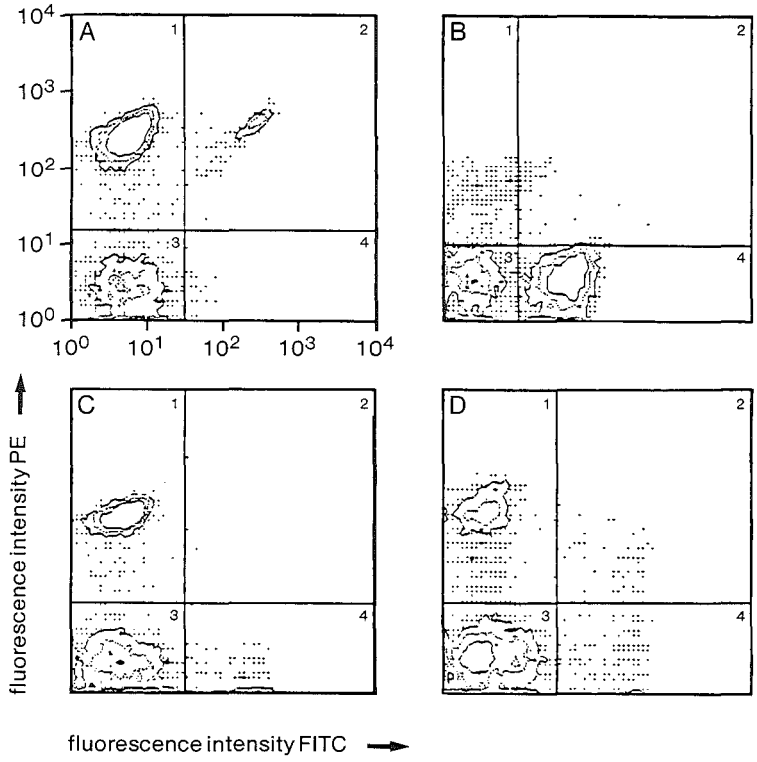
wards K562, but reduced its activity towards JY, Daudi, and MEWO. As expected, anti-TCR-γ/δ-1 had no effect on the activity of clone JS-132.

It is concluded that the MHC-nonrestricted cytotoxicity of TcR-$\gamma\delta^+$ clones 1012 and Wi.K is induced by the culture condition, most likely by growth factors such as IL-2 produced by the feeder cell mixture. Addition of McAb towards TcR-$\gamma\delta$ can specifically induce cytotoxicity.The antibody can also specifically inhibit cytotoxicity, despite the fact that this activity must be categorized as factor induced.

Distribution of TcR-$\gamma(\delta)$-bearing cells in normal PB-MNC and thymus.

Since we had demonstrated the reactivity of anti-TCR-γ/δ-1 McAb with all forms of TcR-$\gamma(\delta)$ described thus far, we felt confident to use this McAb to assess the occurrence of this second type of TcR in PB and thymus of normal individuals. In an initial experiment described above, using FACS analysis of PB-MNC from 18 normal adult donors, variable percentages of TcR-$\gamma\delta^+$ cells had been found, ranging from undetectable to 5%. In a few cases, including that of donor CM, a distinct subpopulation of TcR-$\gamma\delta^+$ cells could be observed. PB-MNC from this donor were used to determine the percentage of $CD3^+$/TcR-$\gamma\delta^+$ cells and the CD4/CD8 phenotype of this population by double immunofluorescence stainings, followed by FACS analysis. As can be seen in Figure 4, 6.7% of $CD3^+$ cells in this individual expressed TcR-$\gamma\delta$ (panel A). The majority of these cells were of the $CD4^-/CD8^-$ phenotype as previously described. No TcR-$\gamma\delta^+$ cells expressing the CD4 marker were detectable (panel C). However, 21% of TcR-$\gamma\delta^+$ cells was $CD8^+$ (panel D). In Figure 4 it is also shown that freshly isolated TcR-$\gamma\delta^+$ cells stain very weakly or not with WT31 McAb (panel B).

To determine the percentage of TcR-$\gamma\delta^+$ cells and their phenotype accurately, PB-MNC samples were labeled by double immunofluorescence stainings and analyzed by fluorescence microscopy. In these cases, PB-MNC were derived from donors ranging in age from 2 mo to 16 y in order to investigate whether age affected the size of the TcR-$\gamma\delta^+$ population. In these samples, the percentages of $CD3^+$, $WT31^+$, and TcR-$\gamma\delta^+$ cells were determined by examination of 1,000 cells for each marker. The CD4/CD8 phenotypes of TcR-$\gamma\delta^+$ cells were determined by examination of 100 TcR-$\gamma\delta^+$ cells. The results from 10 donors are enumerated in Table 4. The percentages of TcR-$\gamma\delta^+$ cells ranged from 1.9 to 7.2% within the $CD3^+$ cell population. The fluorescence of the TcR-$\gamma\delta^+$ population was virtually mutually exclusive with the fluorescence of the $WT31^+$ population. In 4 of 10 donors $CD4^+$/TcR-$\gamma\delta^+$ cells were detected, constituting up to 3.0% of TcR-$\gamma\delta^+$ cells. In all donors but one, $CD8^+$/TcR-$\gamma\delta^+$ cells were found, ranging from 1.0 to 17% of TcR-$\gamma\delta^+$ cells. No obvious correlation between age of the donor and the size of the TcR-$\gamma\delta^+$ population could be found. In addition, Table 4 lists data obtained with thymocytes derived from three children. The relative size of the TcR-$\gamma\delta^+$ population in these thymuses was significantly smaller than in PB-MNC, ranging from 0.1 to 1.0% of $CD3^+$ cells. Also, most TcR-$\gamma\delta^+$ thymocytes were of the $CD4^-/CD8^-$ phenotype, while significantly more $CD8^+$/TcR-$\gamma\delta^+$ than $CD4^+$/TcR-$\gamma\delta^+$ cells were found. The same analysis was done for thymocytes derived from an 18-wk-old fetus. At this early stage in human development, the percentage of TcR-$\gamma\delta^+$ thymocytes was 0.7% of $CD3^+$ cells.



	Relative distribution (%) of the stained PB cells in the four quadrants of the dot plots			
Panels	1	2	3	4
A	64.0	4.58	31.0	0.50
B	3.62	1.07	31.4	63.9
C	45.3	0.01	49.7	4.99
D	20.8	1.14	73.8	4.25

Figure 4. Phenotypic analysis of TcR-$\gamma(\delta)^+$ cells in PB of a healthy donor by double immunofluorescence stainings. PB-MNC were first incubated with anti-TCR-γ/δ-1 McAb, followed by goat anti-mouse Ig-FITC conjugate (A, C, and D) or goat anti-mouse Ig-phycoerythrin conjugate (B). Free binding sites of the second-step reagent were blocked with normal mouse serum and cells were incubated with phycoerythrin-conjugated McAb (A, C, and D) or FITC-conjugated WT31 (B). Fluorescence analysis was performed using a FACStar (Becton Dickinson). Fluorescence intensities for all panels are plotted identically on a ^{10}log scale as indicated in A. **A**: CD3(Leu-4) phycoerythrin-labeled; anti-TCR-γ/δ-1 FITC-labeled. **B**: Anti-TCR-γ/δ-1 phycoerythrin-labeled; WT31 FITC-labeled. **C**: CD4(Leu-3a) phycoerythrin-labeled; anti-TCR-γ/δ-1 FITC-labeled. **D**: CD8(Leu-2a) phycoerythrin-labeled; anti-TCR-γ/δ-1 FITC-labeled. PE, phycoerythrin.

TABLE 4. Percentage and phenotype of anti-TCR-γ/δ-1$^+$ cells in PB of healthy donors and four thymus samples.

Donor	Age	CD3$^+$	WT31$^+$ per CD3$^+$	Anti-TcR-γ/δ-1$^+$ per CD3$^+$	CD4$^+$ per anti-TcR-γ/δ-1$^+$	CD8$^+$ per anti-TcR-γ-δ-1$^+$
PB samples		%	%	%	%	%
HM	2 mo	47	98	1.9	0	10
PvH	2.5 mo	62	98	1.9	3	12
WB	3 mo	38	96	2.4	3	17
KD	13 mo	44	94	5.6	0	9
SB	27 mo	38	95	4.7	0	1
MH	6 y	73	98	1.9	1	5
MJ	7 y	61	90	7.2	0	0
SN	8 y	61	94	3.9	0	6
AvdH	12 y	71	96	4.4	1	1
EH	16 y	63	96	4.1	NT[a]	6
Thymus sample						
A	3 wk	76	99.2	1.0	NT	NT
B	5 y	63	99.5	0.2	0	8
C	10 y	73	99.9	0.1	0	28
D[b]	18 wk[b]	78	99.0	0.7	NT	NT

a. NT, not tested.
b. Fetal thymus.

Ultrastructure of TcR-γ(δ)$^+$ cells in PB.

To study the morphological features of TcR-γ(δ)$^+$ cells in PB, electron microscopy wasused. PB-MNC were derived from a healthy individual, who had previously
been found by FACS analysis to contain a relatively high percentage of TcR-γδ$^+$ cells. To identify these TcR-γδ$^+$ cells in electron microscopy, the total cell population was stained with anti-TCR-γ/δ-1 McAb, followed by rabbit anti-mouse Ig and gold-conjugated protein A. For comparison, cells were labeled in the same fashion using WT31 McAb, which in a freshly isolated PB sample would allow predominantly the identification of TcR-αβ$^+$ cells. The gold labeling was specific as indicated by the fact that 40% of the cells were labeled using WT31 McAb, while 4% of the cells were labeled using anti-TCR-γ/δ-1 McAb, figures corresponding to the results of immunofluorescence stainings. Figure 5 shows two cells, representative of the anti-TCR-γ/δ-1$^+$ population or the WT31$^+$ population. No significant overall differences in ultrastructural morphology between these two populations were observed. Both cell types appeared to be medium-sized lymphocytes, ~7-8 μm in diameter. Moreover, like TcR-αβ$^+$ cells, TcR-γδ$^+$ cells were resting lymphocytes as determined by the following features: a high nucleus/cytoplasm ratio, a single "resting" nucleolus in a heterochromatin-rich nucleus, and a small amount of finely vesicular smooth-surfaced membranes; ribosomes existed as single units rather than as polysomal aggregates; rough endoplasmatic reticulum and Golgi system were almost completely absent and only some small mitochondria and a few lysosomes were observed.

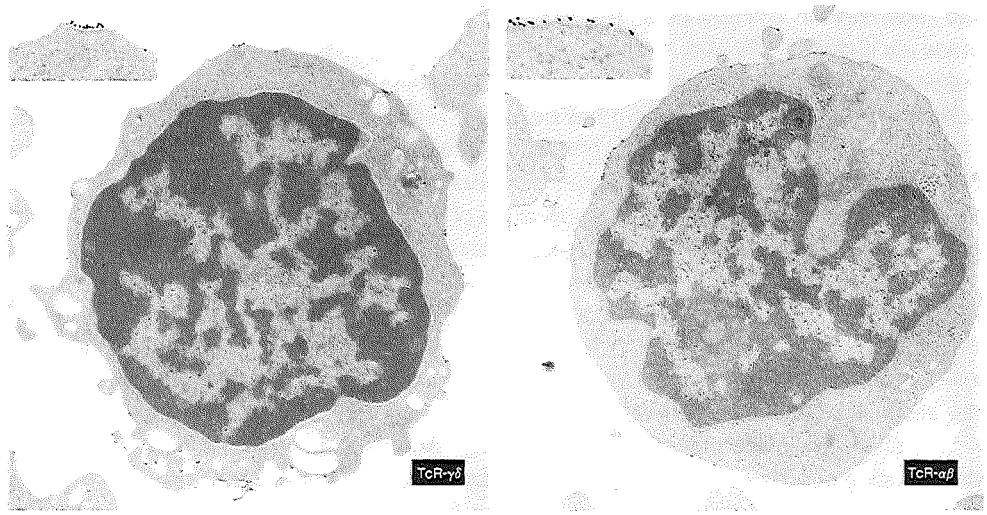


Figure 5. Ultrastructure of TcR-γ(δ)$^+$ cells in PB of a healthy donor. Cells were labeled with either anti-TCR-γ/δ-1 McAb (left) or WT31 McAb (right) and protein A-gold at 4°C in the presence of sodium azide. Cells were subsequently fixed and embedded in Epon. The two insets show a higher magnification (x 16,000) to illustrate labeling of the antigens with gold particles. Bar, 1 μm.

There was no evidence for the presence of significant numbers of "granular structures" as can be identified in activated cytolytic T cells.

DISCUSSION

An McAb specific for human TcR-γ(δ) was generated, using as immunogen CD3/TcR complexes isolated from digitonin-solubilized clones. The major advantage of this McAb over the already available anti-TcR-γ peptide sera is that it reacts with intact, viable cells. This allows positive identification of TcR-γδ-expressing cells by immunofluorescence. Previously, human TcR-γδ$^+$ T cells have been identified using lack of reactivity with WT31 McAb as an indication and immunoprecipitation with anti-Cγ peptide serum as confirmation. This procedure is more time consuming than immunofluorescence, requires a relatively large number of cells, and is not fail-safe, since TcR-γδ$^+$ cells may react with WT31 McAb (see above and reference 27). Reactivity of anti-TCR-γ/δ-1 McAb with viable cells also allows their selective isolation from heterogeneous cell populations and their subsequent use in functional or biochemical studies.

Unlike TcR-αβ, TcR-γ(δ) occurs in different molecular configurations. In the first place, TcR-γ(δ) can occur in either disulfide-linked or non-disulfide-linked forms. The molecular basis of this difference in organization has been unravelled (16,40,41). Of the two C gene segments in the γ locus, Cγ1 encodes a cysteine in its second exon, while Cγ2 does not (11). This cysteine residue is thought to be responsible for the formation of an interchain-disulfide bond (6). Secondly, significant differences in size of TcR-γ chains are detected between non-disulfide-

linked receptors on different cells. The leukemic line PEER (24,39) and the line IDP2 (4,24) express a γ chain of ~55 kDa, while on clones Wi.K and JS-228, as well as on PB-MNC from a leukemic patient (40), we have found a γ chain of 40-44 kDa. In PEER, a triplication of the second exon of the C gene segment has occurred, rather than a duplication as in other cells using Cγ2, which seems to be a polymorphic feature (41). However, since this exon contains only 48 bp, this is not sufficient to explain the size difference between the 55-kDa and the 40-44-kDa γ chains. Posttranslational modification of the γ chain may play a role. A third aspect in which TcR-$\gamma(\delta)$ shows organizational variation, is the appearance of the δ chain. In more than 10 clones expressing disulfide-linked receptors analyzed thus far, we have clearly identified a ~43-kDa δ chain (unpublished results). Clone AK119 is an exception in that no δ chain can be detected after cell surface labeling with ^{125}I. Moingeon et al. (23) have also reported disulfide-linked TcR-γ in which no δ chain could be detected. A similar problem is encountered in the analysis of non-disulfide-linked receptors. In PEER, Weiss et al. (39) observed only a 55-kDa γ chain, while Brenner et al. (24) in addition found a δ chain that migrated at 40 kDa under reducing conditions and at a lower mol. mass under nonreducing conditions. Such a δ chain was also found in the line IDP2 (4). Our results are consistent with those of Brenner et al. In PEER as well as in clone JS-228 derived from PB of a normal individual a δ chain was expressed, which, however, did not label consistently well by cell surface iodination. In clone Wi.K as well as in TcR-γ^+ leukemic T cells (40) no δ chain could be detected by cell surface labeling with ^{125}I. Possibly, the δ chain present in non-disulfidelinked receptors, as well as in the disulfide-linked receptor expressed on clone AK119, cannot be labeled efficiently by this technique. However, to account for such dramatic effect on ^{125}I-labeling this chain must differ significantly in primary structure and/or conformation from the δ chain found in the disulfide-linked receptors. We are currently investigating the occurrence of the δ chain using metabolic labeling. Preliminary results indicate that all types of receptors contain a δ chain. It should be remarked that it is not known, whether the δ chain in disulfide-linked receptors and the δ chain in non-disulfide-linked receptors are products of the same gene.

Anti-TCR-γ/δ-1 McAb reacts with all types of TcR-$\gamma\delta$. It is not clear on which chain of TcR-$\gamma\delta$ the epitope recognized by anti-TCR-γ/δ-1 McAb is located. The McAb does not react in Western blotting, nor after separation of either disulfide-linked or non-disulfide-linked γ and δ chains by reductive alkylation and/or denaturation in SDS at high temperatures. Therefore, this point is difficult to resolve.

TcR-$\gamma\delta$-expressing cells can display MHC-nonrestricted cytotoxicity (21-24,37). As was shown earlier for T cells activated in mixed lymphocyte cultures (42) and cloned TcR-$\alpha\beta^+$ T cells (43), and as is shown here for TcR-$\gamma\delta^+$ T cell clones, such cytolytic activity is induced by the culture conditions. Cells are stimulated weekly with irradiated allogeneic PB-MNC and JY cells, and PHA. Growth factors are produced, such as IL-2, which previously has been shown to enhance cytolytic activity of TcR-$\gamma\delta$-expressing clones (22). A TcR-$\alpha\beta$-expressing clone also displayed MHC-nonrestricted cytotoxicity, which was lost upon factor depletion. However, its specific activity dependent on expression of the HLA-A2 molecule on the target cells, was retained. Similarly, cytotoxicity of TcR-$\gamma\delta$ cells, reflecting the specificity of the receptor, would be expected to remain after factor depletion, particularly since expression of the receptor is not affected. Therefore, TcR-$\gamma\delta$ most likely does not play a role in target cell recognition in the case of *in vitro*-induced MHC-nonrestricted activity, which has also been suggested by others (44).

Recently, evidence has indeed been presented that MHC molecules may be the ligand of TCR-γ/δ (45). However, the receptor plays a role in the regulation of T cell activity in the *in vitro* systems used. McAb directed at the receptor may induce nonspecific cytotoxicity (this paper and ref. 46), and proliferation (23), as shown previously for TcR-$\alpha\beta$expressing cells, which is dependent on the presentation of the McAb via Fc receptors on the target cells (47,48). Also, inhibition of nonspecific cytotoxicity was reproducible found. In one case, cytotoxicity of clone 1012 towards Daudi, which bears Fc receptors for IgG1, was inhibited, while cytotoxicity of clone Wi.K was induced. Perhaps the effect depends on the nature of the conformational change induced in the receptor by binding of the McAb.

It is unknown what contribution TcR-$\gamma\delta^+$ cells make to the immune system. Therefore, it is of interest to determine in which tissues and in what relative numbers TcR-$\gamma\delta^+$ lymphocytes are present in man, in health and disease. In this paper, we have reported the percentages of TcR-$\gamma\delta^+$ cells in PB and thymus of normal individuals. In 11 donors, TcR-$\gamma\delta^+$ cells constituted 1.9 to 7.2% of $CD3^+$ lymphocytes in PB. It has not yet been determined whether these percentages are constant with time in a given healthy donor. In thymus samples from three children, the percentage of TcR-$\gamma\delta^+$ thymocytes ranged from 0.1 to 1.0% of $CD3^+$ cells, which is significantly lower than in PB. These data are consistent with those reported for $CD3^+/WT31^-$ cells in PB-MNC and thymus (38). WT31 expression is indeed very low or non-detectable on freshly isolated TcR-$\gamma\delta^+$ cells. Thymocytes from an 18-wk-old human fetus were included in this study, since Pardoll et al. (49) had reported the expression of TcR-$\gamma\delta$ on all fetal thymocytes at day 15 of gestation in mouse. No higher percentage of TcR-$\gamma\delta^+$ cells were detected in human fetal thymus than in thymuses derived from children. It may be relevant to study earlier samples.

The phenotype of the majority of TcR-$\gamma\delta^+$ cells in PB and thymus was $CD4^-/CD8^-$, in concordance with previously published data. However, in the total pool of TcR-$\gamma\delta^+$ cells, as we have been able to study now, a significant proportion may bear the CD8 antigen, ranging from 0 to 28% between 13 donors, $CD8^+$/TcR-$\gamma\delta^+$ clones have indeed been reported (50). We have found that $CD4^+$/TcR-$\gamma\delta^+$ cells are much more infrequent, but do occur. TcR-$\gamma\delta^+$ cells of the $CD4^+$ phenotype have not previously been reported. In double immunofluorescence stainings using FACS analysis as well as fluorescence microscopy, we have observed some TcR-$\gamma\delta^+$ cells, which did not react with CD3 McAb. Although it is an unlikely possibility, it will have to be investigated whether TcR-$\gamma\delta$ can be expressed at the cell surface in the absence of the CD3 molecule.

Electron microscopic analysis has pointed out that TcR-$\gamma\delta^+$ cells in PB are resting lymphocytes, like TcR-$\alpha\beta^+$ cells and unlike NK cells. This is consistent with our previous observation that $CD3^+/CD4^-/CD8^-$ cells derived from PB need *in vitro* activation in order to become functionally active (51). It will be investigated whether more subtle morphological features discern TcR-$\gamma\delta^+$ cells from TcR-$\alpha\beta^+$ cells. For instance, $CD3^+/CD4^-/CD8^-$ cells have previously been selectively isolated on continuous density gradients, which would imply certain differences in cellular structure (51).

Further studies will involve the identification of human TcR-$\gamma\delta^+$ cells in healthy and diseased states, their isolation and characterization, in which the developed McAb will be an important tool.

ACKNOWLEDGMENTS. We thank J. Sein, W.M. Comans-Bitter, and B. van Krimpen for technical assistance, M. Dessing for FACS analysis, P. Rothengatter for photography, Drs. G.D. Keizer, H. van der Donk, H. Geuze and J.W. Slot for advice and Drs. H.L. Ploegh and C.J.M. Melief for critically reading the manuscript.

This work was supported by the Netherlands Cancer Foundation (J. Borst, E. de Vries and R.J. van de Griend, grants NKI 83-18 and RRTI 84-13) and the Sophia Foundation for Medical Research (J.J.M. van Dongen).

REFERENCES

1. Allison JP, McIntyre BW, Bloch D. Tumor-specific antigen of murine T-lymphoma defined with monoclonal antibody. J Immunol 1982;129:2293-2300.
2. Haskins K, Kubo R, White J, Pigeon M, Kappler J, Marrack P. The major histocompatibility complex-restricted antigen receptor on T cells. I. Isolation with a monoclonal antibody. J Exp Med 1983;157:1149-69.
3. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function: relationship to the T3 molecular complex. J Exp Med 1983;157:705-19.
4. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
5. Lew AM, Pardoll DM, Maloy WL, Fowlkes BJ, Kruisbeek A, Cheng S-F, Germain RN, Bluestone JA, Schwartz RH, Coligan JE. Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. Science 1986;234:1401-5.
6. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Ann Rev Immunol 1986;4:529-91.
7. Saito H, Kranz DM, Takagaki Y, Hayday AC, Eisen HN, Tonegawa S. Complete primary structure of a heterodimeric T-cell receptor deduced from cDNA sequences. Nature 1984;309:757-62.
8. Murre C, Waldmann RA, Morton CC, Bongiovanni KF, Waldmann TA, Shows TB, Seidman JG. Human γ-chain genes are rearranged in leukaemic T cells and map to the short arm of chromosome 7. Nature 1985;316:549-52.
9. Kranz DM, Saito H, Heller M, Takagaki Y, Haas W, Eisen HN, Tonegawa S. Limited diversity of the rearranged T-cell γ gene. Nature 1985;313:752-5.
10. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
11. Strauss WM, Quertermous T, Seidman JG. Measuring the human T cell receptor γ-chain locus. Science 1987;237:1217-9.
12. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-50.
13. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
14. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.
15. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
16. Krangel MS, Band H, Hata S, McLean J, Brenner MB. Structurally divergent human T cell receptor γ proteins encoded by distinct Cγ genes. Science 1987;237:64-7.
17. Chien Y-H, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature 1987;327:677-82.
18. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The δ- and ϵ-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature 1984;312:455-8.
19. Weissman AM, Samelson LE, Klausner RD. A new subunit of the human T-cell antigen receptor complex.

Nature 1986;324:480-2.
20. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, Stobo J. The role of the T3/antigen receptor complex in T-cell activation. Ann Rev Immunol 1986;4:593-619.
21. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocyte. Nature 1987;325:683-8.
22. Van de Griend RJ, Tax WJM, Van Krimpen BA, Vreugdenhil RJ, Ronteltap CPM, Bolhuis RLH. Analysis of tumor cells by $CD3^+4^-8^-16^+$ T cell receptor $\alpha\beta^-$ clones, regulated via CD3 and CD16 activation sites, recombinant interleukin 2, and interferon β. J Immunol 1987;138:1627-33.
23. Moingeon P, Jitsukawa S, Faure F, Troalen F, Triebel F, Graziani M, Forestier F, Bellet D, Bohuon C, Hercend T. A γ-chain complex forms a functional receptor on cloned human lymphocytes with natural killer-like activity. Nature 1987;325:723-6.
24. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 1987;325:689-94.
25. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 1986;322:179-81.
26. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 1985;135:1922-8.
27. Van de Griend RJ, Borst J,Tax WJM, Bolhuis RLH. Functional reactivtiy of WT31 monoclonal antibody with T cell receptor-γ expressing $CD3^+4^-8^-$ T cells. J Immunol 1988;140:1107-10.
28. Borst J, Spits H, Voordouw A, De Vries E, Boylston A, De Vries JE. A family of T-cell receptor molecules expressed on T-cell clones with different specificities for allomajor histocompatibility antigens. Hum Immunol 1986;17:426-42.
29. Van de Griend RJ, Bolhuis RLH. In vitro expansion and analysis of cloned cytotoxic T cells derived from patients with chronic Tγ lymphoproliferative disorders. Blood 1985;65:1002-9.
30. Ang S-L, Seidman JG, Peterman GM, Duby AD, Benjamin D, Lee SJ, Hafler DA. Functional γ chain-associated T cell receptors on cerebrospinal fluid-derived natural killer-like T cell clones. J Exp Med 1987;165:1453-8.
31. Yssel H, De Vries JE, Koken M, Van Blitterswijk W, Spits H. Serum-free medium for generation and propagation of functional human cytotoxic and helper T cell clones. J Immunol Methods 1984;72:219-27.
32. Van de Griend RJ, Bolhuis RLH. Rapid expansion of allospecific cytotoxic T cell clones using nonspecific feeder cell lines without further addition of exogenous IL-2. Transplantation 1984;38:401-6.
33. Ravid Z, Goldblum N, Zaizov R, Schlesinger M, Kertes T, Minowada J, Verbi W, Greaves M. Establishment and characterization of a new leukaemic T-cell line (PEER) with an unusual phenotype. Int J Cancer 1980;25:705-10.
34. Slot JW, Geuze HJ. A new method of preparing gold probes for multiple-labeling cytochemistry. Eur J Cell Biol 1985;38:87-93.
35. Oettgen HC, Pettey CL, Maloy WL, Terhorst C. A T3-like protein complex associated with the antigen receptor on murine T cells. Nature 1986;320:272-5.
36. Brenner MB, Trowbridge IS, Strominger JL. Cross-linking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. Cell 1985;40:183-90.
37. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$, $CD8^-$ T lymphocytes: a CD3-associated disulfide-linked γ chain heterodimer. J Exp Med 1987;165:1076-94.
38. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
39. Weiss A, Newton M, Crommie D. Expression of T3 in association with a molecule distinct from the T-cell antigen receptor heterodimer. Proc Natl Acad Sci USA 1986;83:6998-7002.
40. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-28.
41. Littman DR, Newton M, Crommie D, Ang S-L, Seidman JG, Gettner SN, Weiss A. Characterization of an expressed CD3-associated Ti γ-chain reveals Cγ domain polymorphism. Nature 1987;326:85-8.
42. Bolhuis RLH, Ronteltap CPM, De Rooy-Braam MAW, Van Krimpen BA, Schellekens H. Phytohaemag-

glutinin-induced susceptibility to lysis by natural and activated killer cells of autologous, allogeneic and xenogeneic target cells. Mol Immunol 1982;19:1347-56.

43. Van de Griend RJ, Giphart MJ, Van Krimpen BA, Bolhuis RLH. Human T cell clones exerting multiple cytolytic activities show heterogeneity in susceptibility to inhibition by monoclonal antibodies. J Immunol 1984;133:1222-29.
44. Phillips JH, Weiss A, Gemlo BT, Rayner AA, Lanier LL. Evidence that the T cell antigen receptor may not be involved in cytotoxicity mediated by γ/δ and α/β thymic cell lines. J Exp Med 1987;166:1579-84.
45. Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of $\gamma\delta$ receptor-bearing T lymphocytes. Nature 1987;330:262-4.
46. Moingeon P, Ythier A, Goubin G, Faure F, Nowill A, Delmon L, Rainaud M, Forestier F, Daffos F, Bohuon C, Hercend T. A unique T-cell receptor complex expressed on human fetal lymphocytes displaying natural-killer-like activity. Nature 1986;323:638-40.
47. Spits H, Yssel H, Leeuwenberg J, De Vries JE. Antigen-specific cytotoxic T cell and antigen-specific proliferating T cell clones can be induced to cytolytic activity by monoclonal antibodies against T3. Eur J Immunol 1985;15:88-91.
48. Tax WJM, Hermes FFM, Willems RW, Capel PJA, Koene RAP. Fc receptors for mouse IgG1 on human monocytes: polymorphism and role in antibody-induced T cell proliferation. J Immunol 1984;133:1185-9.
49. Pardoll DM, Fowlkes BJ, Bluestone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
50. Nowill A, Moingeon P, Ythier A, Graziani M, Faure F, Delmon L, Rainaut M, Forrestier F, Bohuon C, Hercend T. Natural killer clones derived from fetal (25 weak) blood: probing the human T cell receptor with WT31 monoclonal antibody. J Exp Med 1986;163:1601-6.
51. Van de Griend RJ, De Bruin HG, Rümke HC, Van Doorn R, Roos D, Astaldi A. Isolation and partial characterization of a novel subset of human T lymphocytes defined by monoclonal antibodies. Immunol 1982;47:313-20.

CHAPTER 4.7

REARRANGEMENT AND EXPRESSION OF T CELL RECEPTOR DELTA GENES IN T CELL ACUTE LYMPHOBLASTIC LEUKEMIAS*

Jacques J.M. van Dongen[1], Ingrid L.M. Wolvers-Tettero[1], Fred Wassenaar[2], Jannie Borst[3] and Peter van den Elsen[2]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam;
2. Department of Immunohematology and Blood Bank, University Hospital, Leiden;
3. Department of Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands.

SUMMARY

We have analyzed T cell receptor δ (TcR-δ) gene rearrangement and transcription in appropriately phenotyped mononuclear cells derived from 12 patients with T cell acute lymphoblastic leukemia (T-ALL). The T-ALL cells were also analyzed for rearrangement and transcription of the TcR-β and γ genes as well as for the presence of TcR-α gene transcripts. Four T-ALL expressed TcR-$\gamma\delta$ at the cell surface, while three expressed TcR-$\alpha\beta$. The other five T-ALL did not express a TcR-CD3 complex on their cell membrane. The TcR-$\gamma\delta^+$ T-ALL had rearranged both TcR-δ gene alleles and contained mature 2.2- and 1.5-kb TcR-δ transcripts. In one case immature 1.9- and 1.2-kb TcR-δ transcripts were also found. Furthermore, they contained mature TcR-γ mRNA, mature or immature TcR-β mRNA, but no TcR-α mRNA. The three TcR-$\alpha\beta^+$ T-ALL contained mature α and β transcripts, but lacked TcR-δ transcripts as a result of deletion of both TcR-δ gene alleles. These data are in line with a mutually exclusive expression of TcR-α and -δ genes, which may be important to ensure the presence of only one type of TcR per T cell.

One out of five CD3$^-$ T-ALL had germline TcR-β, -γ and -δ genes. The other four CD3$^-$ T-ALL had rearranged their TcR-β, -γ and -δ genes and contained immature 1.9- and 1.2-kb TcR-δ gene transcripts. Remarkably, one of these T-ALL also contained TcR-α transcripts in addition to the immature TcR-δ transcripts, which was in line with the deletion of one TcR-δ gene allele and rearrangement of the other allele. This suggests that prevention of dual receptor expression may not only be regulated by the presence of germline TcR-α genes in TcR-$\gamma\delta^+$ cells or by deletion of both TcR-δ gene alleles in TcR-$\alpha\beta^+$ cells, but also via other regulation mechanisms.

---

* *Published in: Blood 1989;74:334-42.*

Finally our data indicated that the combinatorial repertoire of the TcR-δ genes is limited, which has also been described for the TcR-γ genes.

INTRODUCTION

The majority of mature T lymphocytes present in peripheral lymphoid organs, recognize antigens in the context of major histocompatibility (MHC) molecules by means of the classical T cell receptor (TcR), consisting of an α and a β chain (1,2). An alternative TcR, composed of γ and δ chains, has recently been defined in humans (3-5) and mice (6-8). Both receptors are expressed at the cell surface in association with the CD3-protein complex. In normal individuals, TcR-γδ is found on 1 to 10% of mature $CD3^+$ T cells and 0.1 to 1% of $CD3^+$ thymocytes, which predominantly have the $CD4^-/CD8^-$ phenotype (4-12). It has been demonstrated that in vitro activated TcR-$\gamma\delta^+$ T cell clones can exhibit cytotoxic activity and/or secrete growth factors (4,5,9). However, the exact function and specificity of this type of T cells remains to be defined.

All four TcR chains are encoded by genes, which rearrange during differentiation to obtain receptor diversity (13-16). The genomic organization of the human TcR-α, TcR-β and TcR-γ genes has for a large part been unraveled (17-21) and during the last year also many data have become available about the recently discovered TcR-δ genes (22-31). The TcR-δ encoding genes are located within the TcR-α locus, approximately 85 kilobases (kb) 5′ to the Cα genes, in between the long stretch of Jα and the Vα gene segments (14-16,24-30). In addition to three Dδ and three Jδ gene segments, only one Cδ gene has been identified within the murine and human TcR-δ gene complex (14,15,22-31). The TcR-α and TcR-δ gene segments have the same transcriptional orientation, which raises the possibility that α and δ chains can potentially make use of the same V-region-gene pool (14,15,24- 30). Some Vδ genes in the mouse indeed show high homology with previously defined Vα genes (14,15,32) and in humans, the nucleotide sequence of a Vδ-cDNA clone appeared to be virtually identical to a Vα gene of the Vα6-gene family (33).

TcR-δ-gene rearrangements were first detected by use of pulsed-field-gel electrophoresis of large restriction fragments and hybridization with a Cα probe (16). During murine ontogeny, rearrangements in the TcR-δ genes occur at least as early as day 14 in fetal thymocytes (14-16), i.e. at the same time or possibly before rearrangement and transcription of the TcR-γ genes (15,34-36). TcR-β genes start to rearrange from day 14 on, resulting in fully rearranged genes and mature 1.3-kb transcripts two days later (34-38). Rearrangement and transcription of the TcR-α genes occurs from day 17 on (35,36). These data are in line with the finding that TcR-γδ is already expressed by day-14-and day-15-fetal thymocytes, while the first TcR-αβ positive thymocytes can be detected two days later (7,8,39).

Human T cell differentiation as studied by the analysis of TcR gene rearrangement and transcription in T cell acute lymphoblastic leukemias (T-ALL) seems to reflect T cell development during murine ontogeny. This leukemia model system is based on the assumption that T-ALL represent the malignant counterparts of T cells in the various stages of normal differentiation (40,41). It was concluded that TcR-γ genes rearrange and are transcribed before or simultaneously with the TcR-β genes, while TcR-α gene transcription occurs at a later stage during differentiation (42-45).

Here we have used the same model system to analyze TcR-δ gene rearrangement and transcription during human T cell differentiation. Twelve T-ALL samples including four TcR-$\gamma\delta^+$ T-ALL were characterized by extensive immunologic marker analysis and by analysis of the configuration and transcription of the TcR genes.

MATERIALS AND METHODS

Cell samples

Peripheral blood (PB) or bone marrow (BM) samples were obtained from T-ALL patients at initial diagnosis. The cell samples were obtained after informed consent of the patients, according to the guidelines of the Medical Ethics Committee of the University Hospital Dijkzigt/Erasmus University, Rotterdam, The Netherlands. Mononuclear cells (MNC) were obtained from these cell samples by Ficoll-Paque (density: 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. These MNC samples were frozen and stored in liquid nitrogen.

Immunologic marker analysis

The MNC of the T-ALL patients were analyzed for the nuclear expression of terminal deoxynucleotidyl transferase (TdT), for cell membrane expression of the T cell markers CD1(66IIC7), CD2(Leu-5b), CD3(Leu-4), CD4 (Leu-3), CD5(Leu-1), CD7(3A1), and CD8(Leu-2), for the HLA-DR antigen as well as for reactivity with the monoclonal antibody (McAb) WT31, which recognizes a non-polymorphic epitope on TcR-αβ (46). $CD3^+/WT31^-$ T-ALL were tested for reactivity with the recently developed anti-TcR-γδ McAb (anti-TCR-γ/δ-1, hybridoma 11F2; c.f. reference 11), if sufficient cells were available. The rabbit anti-TdT antiserum was obtained from Supertechs (Bethesda, MD); the McAb Leu-1, Leu-2, Leu-3, Leu-4, Leu-5b and the anti-HLA-DR McAb were obtained from Becton Dickinson (Sunnyvale, CA); the 3A1 hybridoma was obtained from the American Type Culture Collection (Rockville, MD). The anti-CD1 antibody and WT31 were gifts from Dr. J.M. van de Rijn (Amsterdam, The Netherlands) and Dr. W. Tax (Nijmegen, The Netherlands), respectively. Indirect immunofluorescence stainings for TdT and the cell membrane markers were performed as described and evaluated with Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, FRG) (47).

Northern blot analysis

Total RNA was isolated from frozen MNC by the LiCl/urea method (48). Approximately 15 μg of total RNA was size-fractionated on 1.0% agarose gels containing formaldehyde and blotted to Biodyne nylon membranes (Pall Ultrafine Filtration Corporation, Glen Core, NY). ^{32}P-nick-translated cDNA probes were used to detect specific RNA sequences for TcR-α (pA65), TcR-β (pT10), TcR-γ(pTγ-1), and CD3-δ (pPGBC9) (18,49-51). The TcR-δ transcripts were detected by use of a recently isolated cDNA probe, which contains exclusively human Cδ sequences (P. van den Elsen, unpublished results).

Southern blot analysis

DNA was isolated from frozen MNC as described (48). Fifteen μg of DNA were digested with the appropriate restriction enzymes, obtained from Boehringer Mannheim (Mannheim, FRG) or from New England Biolabs (Beverly, MA). The restriction fragments were size-fractionated on 0.7% agarose gels and transferred to Gene Screen Plus nylon membranes (New England Nuclear, Boston, MA) or Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, FRG) as described (48).

TcR-γ gene rearrangements were detected with ^{32}P-nick-translated Jγ1.3 and Cγ probes (21,52). The Jγ1.3

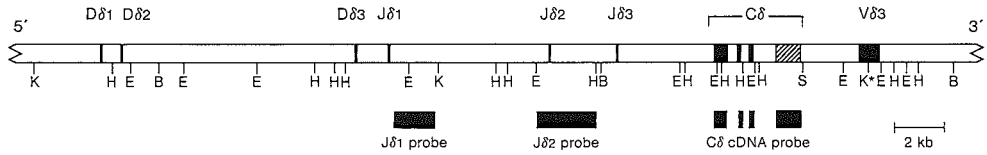


Figure 1. Organization of the D, J and C regions of the human TcR-δ gene (c.f. references 24-31). The locations of the relevant *Kpn*I (K), *Eco*RI (E), *Hin*dIII (H), *Bam*HI (B), and *Sac*I (S) restriction enzyme sites are indicated. K* represents a polymorphic *Kpn*I restriction site. The solid boxes and the shaded box represent the coding exons and the 3′ untranslated Cδ exon, respectively. The solid bars represent the three probes, which were used for hybridization of the Southern blot filters.

probe is a 0.8-kb *Eco*RI-*Hin*dIII fragment containing Jγ1.3 segments; it hybridizes to both Jγ1.3 and Jγ2.3 gene segments and allows detection of rearrangements in these regions in *Eco*RI digests (21). In *Kpn*I digests the Jγ1.3 probe detects each rearrangement in the Jγ1 and Jγ2 regions (53). The Cγ probe is a 0.4-kb *Bam*HI-*Bam*HI fragment containing a part of the first exon of the Cγ1 gene; it hybridizes to the first exon of Cγ1 and Cγ2 and allows detection of rearrangements to the γ1 and γ2 locus in *Bam*HI digests (21). Deletion of Cγ1 gene segments can be detected by this probe in *Eco*RI digests (21,52). All 12 T-ALL DNA samples were analyzed with the Jγ1.3 probe (*Eco*RI and *Kpn*I digests) and the Cγ probe (*Bam*HI and *Eco*RI digests).

The configuration of the TcR-β genes was analyzed with Cβ and Jβ2 probes (54). The Cβ probe is a 0.7-kb *Pst*I-*Bgl*II fragment containing Cβ1 segments; it hybridizes to both Cβ1 and Cβ2 gene segments and allows detection of rearrangements to the β1 locus in *Eco*RI digests (54). The Jβ2 probe consists of contiguous 1.9-kb *Pvu*II-*Pvu*II and 1.5-kb *Pvu*II-*Eco*RV fragments containing Jβ2.3 to Jβ2.7; this probe does not hybridize to Jβ1 gene segments and was used to detect rearrangements to the β2 locus in *Eco*RI digests (54). TcR-β gene rearrangements were confirmed using the Cβ probe in *Bam*HI digests.

The configuration of the TcR-δ genes was analyzed by use of the Jδ1 probe JδS16 (a 1.5-kb *Sac*I fragment) and the Jδ2 probe R21XH (a 2.3-kb *Hin*dIII-*Eco*RI fragment) (24,25) as well as by use of the above mentioned cDNA probe, which recognizes Cδ sequences only. All twelve DNA samples were analyzed by use of these probes in *Kpn*I, *Eco*RI, *Bgl*II, *Hin*dIII, and *Bam*HI digests as well as in *Kpn*I/*Hin*dIII, *Kpn*I/*Bam*HI and *Bam*HI/*Sac*I double digests. The three TcR-δ probes and the relevant *Kpn*I, *Bam*HI, *Eco*RI, *Hin*dIII and *Sac*I sites are indicated in Figure 1. The location of the restriction sites in Figure 1 are derived from data in the literature (25-31) as well as from extensive Southern blot analysis of germline TcR-δ genes in normal individuals using the three TcR-δ probes.

Recombinant plasmids were handled under PZEK1 containments according to the National Institutes of Health Guidelines for Research Involving Recombinant DNA molecules.

RESULTS

Phenotype of the ALL

Of the 12 T-ALL analyzed, 7 expressed CD3 on the cell membrane, indicative of TcR surface expression (Table 1, patients 6 through 12). Of these, three were TcR-αβ positive (patients 10 through 12), as determined by immunofluorescence with the McAb WT31, while four were TcR-γδ positive (patients 6 through 9), as determined by immunofluorescence with the McAb anti-TCR-γ/δ-1 (11) and/or previously reported immunoprecipitation studies (52). Two TcR-$\gamma\delta^+$ T-ALL had the expected $CD1^-/CD4^-/CD8^-$ phenotype, while two expressed CD1 and CD4. For further details on the marker analysis, we refer to Table 1.

TABLE 1. Immunologic marker analysis, Southern-blot analysis and Northern blot analysis of twelve T-ALL cell samples

Cell sample	1 BM	2 PB	3 PB	4 PB	5 PB	6 PB	7 PB	8 PB	9 PB	10 PB	11 BM	12 PB
Immunologic markers[a]												
TdT	+	+	+	+	+	+	+	+	+	+	+	-
HLA-DR (L243)	+	-	-	-	-	-	-	18%	-	-	-	-
CD1 (66IIC7)	-	-	+	66%	+	26%	40%	-	-	52%	-	-
CD2 (Leu-5b)	+	+	50%	37%	+	+	+	+	+	+	+	+
CD3 (Leu-4)	-	-	-	-	-	52%	+[b]	+[b]	+[b]	71%	+	+
CD4 (Leu-3)	-	-	+	+	-	38%	59%	-	-	65%	48%	+
CD5 (Leu-1)	-	+	+	+	+	+	+	+	+	+	+	+
CD7 (3A1)	+	+	+	+	+	+	+	+	+	+	+	+
CD8 (Leu-2)	-	-	44%	67%	+	-	-	-	-	+	32%	+
TcR-αβ (WT31)	-	-	-	-	-	-	-[b]	-[b]	-[b]	+	+	+
TcR-γδ (11F2)	NT[c]	NT	NT	NT	NT	+	+[b]	+[b]	NT[b]	NT	NT	NT
Southern blot analysis[d]												
TcR-β1 genes	G/G	R/R	D/R	D/R	R/G	R/G	D/D	R/G	D/R	D/R	D/R	D/R
TcR-β2 genes	G/G	R/G	R/R	R/R	R/G	R/R	R/R	G/G	R/G	R/R	R/G	R/R
TcR-γ1 genes	G/G[e]	D/D	D/D	D/D	D/D	D/D	D/D	R/R	D/D	D/D	D/D	R/G[f]
TcR-γ2 genes	G/G	R/R[g]	R/R	R/R	R/R	R/R	R/R	G/G	R/R	R/R	R/R[g]	G/G
TcR-δ genes[h]	G/G	D/R	R/R	R/R	R/R	R/R	R/R	R/R	R/R	D/D	D/D	D/D
Northern blot analysis[i]												
TcR-α (1.7 kb)	-	±	-	-	-	-	-	-	-	+	+	+
TcR-β (1.3 kb)	-	+	+	+	+	-	+	-	-	+	+	+
TcR-β (1.1 kb)	+	+	-	-	-	+	-	-	-	-	-	-
TcR-β (1.0 kb)	-	-	±	-	±	-	±	+	+	-	-	±
TcR-γ (1.6 kb)	-	±	+	+	-	+	+	+	+	-	±	-
TcR-δ (2.2 kb)	-	-	-	-	-	+	+	+	+	-	-	-
TcR-δ (1.9 kb)	-	±	+	+	+	-	-	+	-	-	-	-
TcR-δ (1.5 kb)	-	-	-	-	-	+	+	+	+	-	-	-
TcR-δ (1.2 kb)	-	±	±	±	±	-	-	+	-	-	-	-
CD3-δ	+	+	+	+	+	+	+	+	+	+	+	+

a. Immunologic marker analysis: +, more than 75% of the cells is positive; -, less than 15% of the cells is positive; percentages positivity between 15% and 75% are indicated.
b. By immunoprecipitation it was proven that the $CD3^+$ T-ALL cells of patients 7, 8 and 9 expressed TcR-γδ (c.f. reference 52); the T-ALL cells of patient 8 expressed disulfide-linked TcR chains, whereas the T-ALL cells of patients 7 and 9 expressed a TcR without interchain disulfide bond.
c. NT, not tested.
d. Southern blot analysis: G, allele in germline configuration; R, rearranged allele; D, deletion of the involved gene complex.
e. The T-ALL cells of patient 1 had germline TcR-γ genes; also no rearrangement to the Jγ1.1 region could be detected.
f. The TcR-γ genes of the T-ALL cells of patient 12 were rearranged to Jγ1.1 on one allele, but the other allele was in germline configuration.
g. In the T-ALL cells of patients 2 and 11 one TcR-γ2 allele was rearranged to Jγ2.3, while the other allele was probably rearranged to Jγ2.1.
h. Detailed information concerning the configuration of the TcR-δ genes in the twelve T-ALL is summarized in Table 2.
i. Northern blot analysis: +, transcripts present; ±, weak band visible; -, no transcripts could be detected.

TcR gene rearrangements in the T-ALL

All T-ALL with the exception of the so-called prothymocytic T-ALL (patient 1; c.f. ref. 55) had rearranged TcR-β genes. In the majority of cases this concerned rearrangement of both alleles and involvement of the β2 locus. TcR-γ gene rearrangements had also occurred in all but the prothymocytic T-ALL. In all these cases, except the TcR-$\gamma\delta^+$ T-ALL of patient 8 and the TcR-$\alpha\beta^+$ T-ALL of patient 12, both Cγ1 alleles were deleted, while rearrangements had taken place to the γ2 locus on both alleles. Further details concerning the configuration of the TcR-β and TcR-γ genes are summarized in Table 1.

Both TcR-δ gene alleles were deleted in the three TcR-$\alpha\beta^+$ T-ALL, as would be expected to occur upon TcR-α gene rearrangement (14,16,25,26). TcR-δ gene rearrangements were found in all four TcR-$\gamma\delta^+$ T-ALL as well as in four out of five CD3$^-$ T-ALL. Based on the extensive analyses of the TcR-δ genes by use of eight different restriction enzyme digests and the three described TcR-δ probes, it appeared to be possible to determine the configuration of the TcR-δ genes in the eight T-ALL with rearranged δ genes (Table 2). In the T-ALL of patient 2, one TcR-δ gene allele was deleted, while the other was rearranged. In most T-ALL the TcR-δ gene alleles were rearranged to the Jδ1 gene segment, but in two T-ALL, a rearrangement to the Jδ2 gene segment was found. Rearrangements to the Jδ1 gene segment could be identified in *Eco*RI and *Bgl*II digests using the Jδ1 probe (Figure 2A). These rearrangements were confirmed by use of several other digests, including *Kpn*I and *Bam*HI. Rearrangements to the Jδ2 gene segment could be detected in *Hin*dIII and *Bgl*II digests using the Jδ2 probe and were confirmed in several other digests, including *Kpn*I and *Kpn*I/*Bam*HI digests (Figure 2B). It should be remarked that our analyses of the leukemic T cells and cells with germline TcR-δ genes indicated that the *Kpn*I site at the 3´ site of the C gene segments (located in the Vδ2 gene segment) is polymorphic. Therefore *Kpn*I digests will not always be informative and should be carefully interpreted.

TABLE 2. Configuration of the TcR-δ genes: summary of the results of the Southern blot analysis using eight different restriction enzyme digests and the three described TcR-δ probes.

Patients	1	2	3	4	5	6	7	8	9	10	11	12
Jδ1 gene segment	G/G[a]	D/R[a]	R/R	D/R	R/R	R/R	D/R or R/R[c]	R/R	R/R	D/D	D/D	D/D
Jδ2 gene segment	G/G	D/G	R/?[b]	R/G	G/G	G/G	G/G	G/G	G/G	D/D	D/D	D/D
Jδ3 gene segment	G/G	D/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	D/D	D/D	D/D
Cδgene segment	G/G	D/G	G/G	G/G	G/G	G/G	G/G	G/G	G/G	D/D	D/D	D/D
Vδ2 gene segment	G/G	D/R	R/G	G/G	R/G	G/G	G/G	G/G	R/G	D/D	D/D	D/D
Combined results	G/G	D/R	R/R	R/R	R/R	R/R	R/R	R/R	R/R	D/D	D/D	D/D

a. G, allele in germline configuration; R, rearranged allele; D, deletion of the involved gene (segment).
b. In some digests of the DNA from patient 3 hybridization of the Southern blot filters with the Jδ2 probe showed two rearranged bands, while in other digests only one rearranged band was seen (c.f. Figure 2B).
c. In the T-ALL cells from patient 7 most probably the two rearranged TcR-δ gene alleles are rearranged identically as detected by the various TcR-δ probes.

In four T-ALL, including the TcR-$\gamma\delta^+$ T-ALL of patient 9, one of the TcR-δ gene alleles appeared to be rearranged to the Vδ2 gene segment, which is located within 3 kb at the 3′ side of the Cδ region (31). Rearrangements to the Vδ2 gene segment could be detected in *Bgl*II, *Bam*HI and most *Kpn*I digests using the Cδ probe (Figure 2C). In case of rearrangements detected in *Kpn*I and *Bam*HI digests, possible rearrangements to the Jδ2 and/or Jδ3 gene segments were studied by use of a *Bam*HI/*Sac*I double digest and the Cδ probe and by use of a *Kpn*I/*Bam*HI double digest and the Jδ2 probe. Our Southern blot analyses indicate that rearrangements to the Vδ2 gene segment can be detected in *Bgl*II digests using the Cδ probe (Figure 2C).

Transcription of the CD3 and TcR genes

CD3-δ transcripts were present in all 12 T-ALL, supporting their T cell origin (42,43,56). TcR-α transcripts were detected in the three TcR-$\alpha\beta^+$ T-ALL. A weak 1.7-kb TcR-α band was also found in one $CD3^-$ T-ALL (patient 2), which may be due to the 3% contaminating normal T cells in the sample (Table 1). However, it most probably concerns α transcripts of the leukemic cells, since in this T-ALL one TcR-δ gene allele appeared to be rearranged, while the other was probably deleted, suggesting that a TcR-α gene rearrangement had occurred (Table 2; Figure 2).

Mature 1.3-kb TcR-β transcripts were detected in the three TcR-$\alpha\beta^+$ T-ALL, in the $CD3^-$ T-ALL except for the prothymocytic T-ALL, and in one out of four TcR-$\gamma\delta^+$ T-ALL. Immature 1.0-kb TcR-β transcripts, which are probably derived from genes with D-J joining, were found in one TcR-$\alpha\beta^+$ T-ALL, in three out of four TcR-$\gamma\delta^+$ T-ALL and in two $CD3^-$ T-ALL. Immature 1.1-kb TcR-β transcripts, which may be derived from germline TcR-β genes (42,43), were found in the prothymocytic T-ALL, in one other $CD3^-$ T-ALL and in one TcR-$\gamma\delta^+$ T-ALL.

The TcR-γ gene was expressed in all four TcR-$\gamma\delta^+$ T-ALL, in three out of five $CD3^-$ T-ALL, and at a low level in one out of three TcR-$\alpha\beta^+$ T-ALL. One $CD3^-$ T-ALL, in which extensive γ gene rearrangement had occurred, did not express mature γ mRNA (patient 5).

Four different types of TcR-δ gene transcripts have been described (Figure 3). The 1.9- and 1.2-kb transcripts do not contain V-region sequences and thus are probably derived from incomplete rearranged TcR-δ genes, while the 2.2- and 1.5-kb transcripts do contain V regions (22,23). The occurrence of two types of mature and immature δ transcripts is probably caused by variability in the site of polyadenylation (22,23). TcR-δ mRNA of 2.2 and 1.5 kb was present in all four TcR-$\gamma\delta^+$ T-ALL, while no TcR-δ transcripts were detected in the TcR-$\alpha\beta^+$ T-ALL (Figure 3). In all $CD3^-$ T-ALL except of the prothymocytic leukemia only immature 1.9- and 1.2-kb transcripts were found (Figure 3), indicating that the transcribed TcR-δ genes in these T-ALL are rearranged incompletely (patient 2, 3, 4 and 5). Interestingly, in one of the TcR-$\gamma\delta^+$ samples (patient 8) also both types of immature transcripts were detected in addition to the mature TcR-δ transcripts, which denotes that one of the TcR-δ gene alleles is rearranged incompletely (Figure 3).

A
kb
8.8 -
8.0 -
5.8 -
5.4 -
5.1 -
4.7 -
- G
patient 1
patient 2
patient 3
patient 4
patient 5
patient 6
patient 7
patient 8
patient 9
patient 10
patient 11
patient 12
control
BglII, Jδ1 probe
B
kb
3.5 >
3.4 -
2.8 -
- G
patient 1
patient 2
patient 3
patient 4
patient 5
HindIII, Jδ2 probe
kb
7.1 -
6.4 -
4.3 -
3.7 -
- G
patient 1
patient 2
patient 3
patient 4
patient 5
KpnI/BamHI, Jδ2 probe

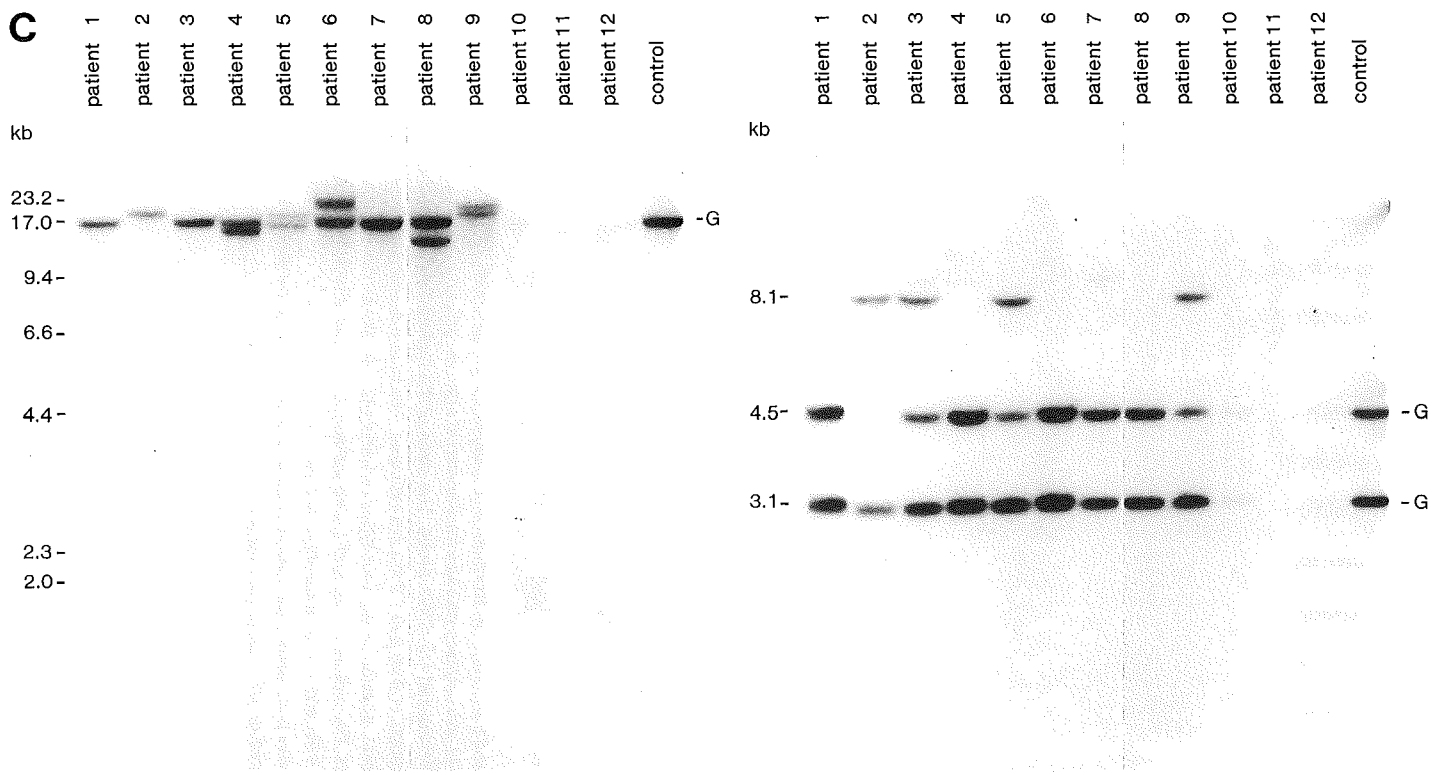


Figure 2. Southern blot analysis of the TcR-δ genes. DNA samples from the T-ALL and from a B cell leukemia as a control were digested with *Bgl*II (**A** and **C**), *Hin*dIII (**B**), *Kpn*I/*Bam*HI (**B**) or *Kpn*I (**C**). The restriction fragments were size fractionated on agarose gels and transferred to nylon filters. The filters were hybridized with the ^{32}P-labeled Jδ1 probe (**A**), Jδ2 probe (**B**), or Cδ probe (**C**). The sizes (in kb) of the germline bands (G) and the majority of rearranged bands are indicated.

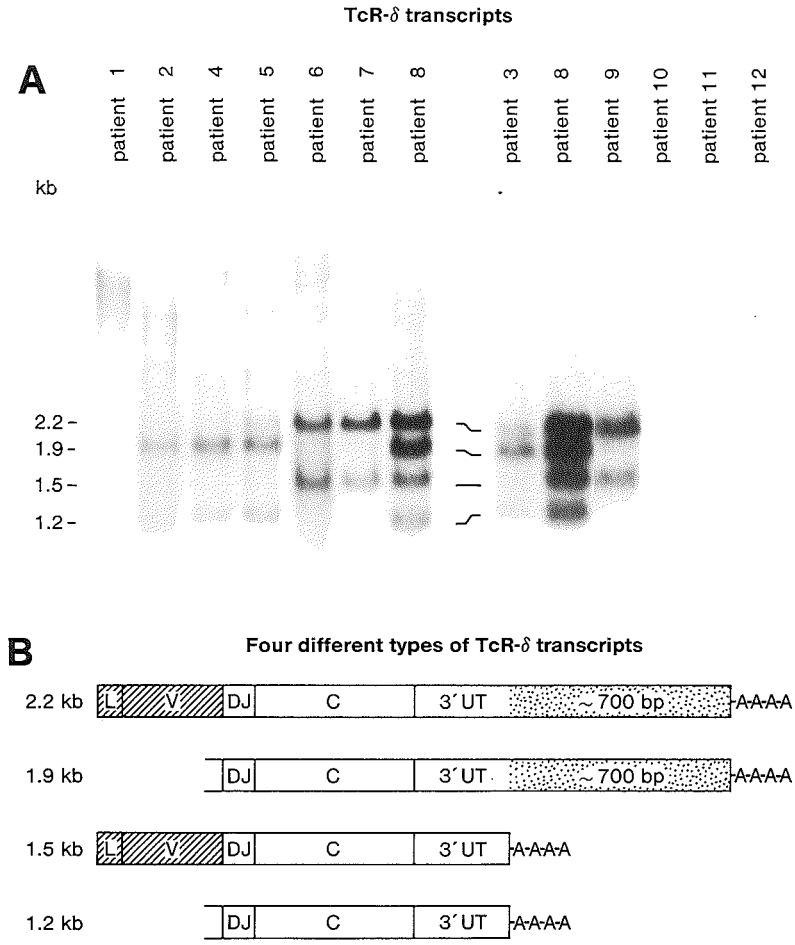


Figure 3. TcR-δ gene transcripts. **A**: Northern blot analysis for the detection of TcR-δ gene transcripts. Total RNA from the twelve T-ALL cell samples was size fractionated in agarose gels and blotted to nylon filters. The filters were hybridized to the ^{32}P-labeled Cδ cDNA probe. The sizes of the mature TcR-δ transcripts (2.2 kb and 1.5 kb) and the immature transcripts (1.9 kb and 1.2 kb) are indicated. The RNA from patient 8 was used in both Northern blots to indicate the precise position of the four types of transcripts. **B**: Schematic diagram of the four different types of TcR-δ transcripts. The mature 2.2-kb and 1.5-kb transcripts contain V regions (shaded area), which are lacking in the immature 1.9-kb and 1.2-kb transcripts (22,23). The occurrence of two types of mature and immature δ transcripts (γ700 base pairs difference; dotted area) is probably caused by variability in the site of polyadenylation in the 3′ untranslated (3′UT) sequences (22,23).

DISCUSSION

We have analyzed 12 T-ALL samples for TcR gene rearrangement and expression, of which four expressed TcR-$\gamma\delta$ at the cell surface and three TcR-$\alpha\beta$. The other five T-ALL did not express a TcR-CD3 complex. It should be mentioned that this series is not representative of all T-ALL, but has been selected in part for CD3$^+$/TcR-$\alpha\beta^-$ T-ALL. The real frequency of TcR-$\gamma\delta^+$

T-ALL is probably much lower, which is in line with the low frequency of TcR-$\gamma\delta^+$ cells in normal PB and thymus (11,12).

In the four TcR-$\gamma\delta^+$ T-ALL, both TcR-δ gene alleles appeared to be rearranged. They all contained mature 2.2- and 1.5-kb TcR-δ transcripts as well as TcR-γ transcripts, but lacked TcR-α gene transcripts. One TcR-$\gamma\delta^+$ T-ALL contained immature 1.9- and 1.2-kb transcripts, indicating that the rearrangement of one TcR-δ gene allele was incomplete, while the other was rearranged functionally.

TcR-δ gene rearrangements were not only found in the TcR-$\gamma\delta^+$ T-ALL, but also in four out of five CD3$^-$ T-ALL. The majority of the rearranged TcR-δ genes appeared to be rearranged to the Jδ1 gene segment. In two CD3$^-$ T-ALL a rearrangement to the Jδ2 gene segment was found, while rearrangements to the Jδ3 gene segment were not detected in this series of T-ALL. Rearrangements to the Vδ2 gene, which is located at the 3´ side of the Cδ gene, were found on one allele in a TcR-$\gamma\delta^+$ T-ALL as well as in three CD3$^-$ T-ALL. Also, in adult murine thymocytes, rearrangement to the Vδ gene at the 3´ side of the Cδ region often occurs (57). These combined data suggest that the combinatorial diversity of the TcR-δ genes is limited, which is illustrated by the restricted number of differently rearranged bands in Figure 2. This is in line with the fact that only six Vδ, three Dδ, and three Jδ gene segments have been reported so far (25,29,31,58,59). Such a limited combinatorial diversity has also been described for the TcR-γ genes (21,60,61). On the other hand, an extensive junctional diversity has been found in both TcR-γ and TcR-δ genes (15,31,32,62,63), resulting in an enormous diversity of TcR-$\gamma\delta$ receptors (32).

High levels of TcR-α mRNA were only found in the three TcR-$\alpha\beta^+$ T-ALL. In these T-ALL, the TcR-δ genes had been deleted, probably due to TcR-α gene rearrangement (14,16,25,26). Consequently, they did not contain any TcR-δ gene transcripts. TcR-α and TcR-δ proteins have certain structural homologies, while also β and γ proteins have certain common properties, which are considered important for TcR-chain pairing (14). Therefore, most likely α and δ chains will not pair with each other, nor will β and γ chains. The latter has indeed be shown in the cell line PEER, where β and γ proteins are expressed simultaneously; in this cell line a δ protein is present, which exclusively pairs with the γ chain (64). Apparently β, γ and δ gene rearrangement and expression can and do overlap without leading to aberrant TcR protein expression. There obviously exists an exclusion mechanism to avoid dual receptor expression. Deletion of TcR-δ genes in TcR-$\alpha\beta^+$ cells is in line with such an exclusion mechanism (14,16,22,25,26).

So far only limited data are available about the TcR-δ genes in the postulated hierarchy of human TcR gene rearrangement. Recently two immature T-ALL with rearranged TcR-δ genes, but germline β and γ genes have been reported, suggesting that TcR-δ genes may rearrange early during T cell differentiation (65). This would be in line with the murine ontogenic data (14-16), which indicate that TcR-$\gamma\delta^+$ thymocytes develop first and that TcR-$\alpha\beta^+$ thymocytes arise two days later (7,8,39). Several studies suggest that T cells which have non-functional TcR-γ and/or TcR-δ gene rearrangements can still rearrange their TcR-β and TcR-α genes and develop into TcR-$\alpha\beta^+$ cells (7,52). The series of T-ALL presented here fits within such a differentiation model. However, this series does not add new information with respect to the hierarchy of TcR gene rearrangement, because four CD3$^-$ T-ALL had rearranged their TcR-β, -γ and -δ genes, while these genes were in germline configuration in the fifth CD3$^-$ T-ALL. On the other hand, the CD3$^-$ leukemia of patient 2 is interesting, because this leukemia may represent a

transient cell between the TcR-$\gamma\delta$ lineage and the TcR-$\alpha\beta$ lineage. The leukemic cells of this patient do not (yet) express a TcR-$\alpha\beta$ on the membrane, but already contain mature TcR-β and low amounts of TcR-α transcripts, while also TcR-γ and immature TcR-δ transcripts are present. This suggests that exclusion of dual receptor expression can not only be regulated by the presence of germline TcR-α genes in TcR-$\gamma\delta^+$ cells or deletion of both TcR-δ gene alleles in TcR-$\alpha\beta^+$ cells, but that also other regulation mechanisms may be involved. Future studies have to elucidate whether prevention of co-expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ can also be regulated at the RNA or protein level.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof. Dr R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Dr. T. Rabbitts for his advice and suggestions as well as for providing the Jδ1 and Jδ2 probes, Ms. W.M. Comans-Bitter for her skilful technical assistance, Ms. A. Wicherink for critical reading of the manuscript, Prof. Dr. J. Abels, Dr. K. Hählen, Dr. A.C.J.M. Holdrinet, Dr. J.C. Kluin-Nelemans, Dr. J.J. Michiels and Dr. G.E. van Zanen for providing the T-ALL cell samples, Mr. C. van Dijk, Mr. V.T.M. Gribling (Audiovisueel Centrum of the Erasmus University) and Mr. T.M. van Os for their photographic assistance, Dr. A.C. Duby, Dr. C. Murre and Dr. T. Quertermous for kindly providing genomic and cDNA probes, and Ms. J. de Goey-Van Dooren for typing this manuscript.

This work was supported by the Netherlands Organization for Scientific Research (F. Wassenaar and P. van den Elsen, grant H90-92; J. Borst, grant H93-155).

REFERENCES

1. Meuer SC, Acuto O, Hercend T, Schlossman SF, Reinherz EL. The human T-cell receptor. Annu Rev Immunol 1984;2:23-50.
2. Allison JP, Lanier LL. Structure, function, and serology of the T-cell antigen receptor complex. Annu Rev Immunol 1987;5:503-40.
3. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
4. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325:683-8.
5. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 1987;325:689-94.
6. Lew AM, Pardoll DM, Maloy WL, Fowlkes BJ, Kruisbeek A, Cheng S-F, Germain RN, Bluestone JA, Schwartz RH, Coligan JE. Characterization of T cell receptor gamma chain expression in a subset of murine thymocytes. Science 1986;234:1401-5.
7. Pardoll DM, Fowlkes BJ, Bluestone JA, Kruisbeek A, Maloy WL, Coligan JE, Schwartz RH. Differential expression of two distinct T-cell receptors during thymocyte development. Nature 1987;326:79-81.
8. Bluestone JA, Pardoll D, Sharrow SO, Fowlkes BJ. Characterization of murine thymocytes with CD3-associated T-cell receptor structures. Nature 1987;326:82-4.
9. Marušić-Galešić S, Pardoll DM, Saito T, Leo O, Fowlkes BJ, Coligan J, Germain RN, Schwartz RH, Kruisbeek AM. Activation properties of the T cell receptor-$\gamma\delta$ hybridomas expressing diversity in both γ- and δ-chains. J Immunol 1988;140:411-8.
10. Lanier LL, Federspiel NA, Ruitenberg JJ, Phillips JH, Allison JP, Littman D, Weiss A. The T cell antigen receptor complex expressed on normal peripheral blood $CD4^-$, $CD8^-$ T lymphocytes. J Exp Med 1987;165:1076-94.
11. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.

12. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
13. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
14. Chien Y, Iwashima M, Kaplan KB, Elliott JF, Davis MM. A new T-cell receptor gene located within the alpha locus and expressed early in T-cell differentiation. Nature 1987;327:677-82.
15. Chien Y, Iwashima M, Wettstein DA, Kaplan KB, Elliott JF, Born W, Davis MM. T-cell receptor δ gene rearrangements in early thymocytes. Nature 1987;330:722-7.
16. Lindsten T, Fowlkes BJ, Samelson LE, Davis MM, Chien Y-H. Transient rearrangements of the T cell antigen receptor α locus in early thymocytes. J Exp Med 1987;166:761-75.
17. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.
18. Duby AD, Klein KA, Murre C, Seidman JG. A novel mechanism of somatic rearrangement predicted by a human T-cell antigen receptor β-chain complementary DNA. Science 1985;228:1204-6.
19. Toyonaga B, Yoshikai Y, Vadasz V, Chin B, Mak TW. Organization and sequences of the diversity, joining, and constant region genes of the human T-cell receptor β-chain. Proc Natl Acad Sci USA 1985;82:8624-8.
20. LeFranc M-P, Forster A, Rabbitts TH. Genetic polymorphism and exon changes of the constant regions of the human T-cell rearranging gene γ. Proc Natl Acad Sci USA 1986;83:9596-9600.
21. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
22. Hata S, Brenner MB, Krangel MS. Identification of putative human T cell receptor δ complementary DNA clones. Science 1987;238:678-82.
23. Loh EY, Lanier LL, Turck CW, Littman DR, Davis MM, Chien Y-H, Weiss A. Identification and sequence of a fourth human T cell antigen receptor chain. Nature 1987;330:569-76.
24. Boehm T, Baer R, Lavenir I, Forster A, Waters JJ, Nacheva E, Rabbitts TH. The mechanism of chromosomal translocation t(11;14) involving the T-cell receptor Cδ locus on human chromosome 14q11 and a transcribed region of chromosome 11p15. EMBO J 1988;7:385-94.
25. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-8.
26. Takihara Y, Champagne E, Griesser H, Kimura N, Tkachuk D, Reimann J, Okada A, Alt FW, Chess L, Minden M, Mak TW. Sequence and organization of the human T cell δ chain gene. Eur J Immunol 1988;18:283-7.
27. Griesser H, Champagne E, Tkachuk D, Takihara Y, Lalande M, Baillie E, Minden M, Mak TW. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. Eur J Immunol 1988;18:641-4.
28. Isobe M, Russo G, Haluska FG, Croce CM. Cloning of the gene encoding the δ subunit of the human T-cell receptor reveals its physical organization within the α-subunit locus and its involvement in chromosome translocations in T-cell malignancy. Proc Natl Acad Sci USA 1988;85:3933-7.
29. Takihara Y, Tkachuk D, Michalopoulos E, Champagne E, Reimann J, Minden M, Mak TW. Sequence and organization of the diversity, joining, and constant region genes of the human T-cell δ-chain locus. Proc Natl Acad Sci USA 1988;85:6097-6101.
30. Satyanarayana K, Hata S, Devlin P, Roncarolo MG, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-70.
31. Loh EY, Cwirla S, Serafini AT, Philips JH, Lanier LL. The human T-cell receptor delta chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
32. Elliott JF, Rock EP, Patten PA, Davis MM, Chien Y. The adult T-cell receptor δ-chain is diverse and distinct from that of fetal thymocytes. Nature 1988;331:627-31.
33. Guglielmi P, Davi F, D'Auriol L, Bories J-C, Dausset J, Bensussan A. Use of a variable α region to create a functional T-cell receptor δ chain. Proc Natl Acad Sci USA 1988;85:5634-8.
34. Born W, Rathbun G, Tucker P, Marrack P, Kappler J. Synchronized rearrangement of T-cell γ and β chain genes in fetal thymocyte development. Science 1986;234:479-82.
35. Raulet DH, Garman RD, Saito H, Tonegawa S. Developmental regulation of T-cell receptor gene expression. Nature 1985;314:103-7.

36. Snodgrass HR, Dembić Z, Steinmetz M, Von Boehmer H. Expression of T-cell antigen receptor genes during fetal development in the thymus. Nature 1985;315:232-3.
37. Snodgrass HR, Kisielow P, Kiefer M, Steinmetz M, Von Boehmer H. Ontogeny of the T-cell antigen receptor within the thymus. Nature 1985;313:592-5.
38. Born W, Yagüe J, Palmer E, Kappler J, Marrack P. Rearrangement of T-cell receptor β-chain genes during T-cell development. Proc Natl Acad Sci USA 1985;82:2925-9.
39. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 1988;335:443-5.
40. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
41. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukaemias and non-Hodgkin's lymphomas. Immunological markers and their CD codes. Neth J Med 1988;33:298-314.
42. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
43. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
44. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ: T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
45. Boehm TLJ, Werle A, Ganser A, Kornhuber B, Drahovsky D: T cell receptor γ chain variable gene rearrangements in acute lymphoblastic leukemias of T and B lineage. Eur J Immunol 1987;17:1593-7.
46. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE: Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 1985;135:1922-8.
47. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.
48. Maniatis T, Fritsch EF, Sambrook J. Molecular cloning: a laboratory manual. New York: Cold Spring Harbor Laboratory, 1982.
49. Sim GK, Yagüe J, Nelson J, Marrack P, Palmer E, Augustin A, Kappler J: Primary structure of human T-cell receptor α-chain. Nature 1984;312:771-5.
50. Dialynas DP, Murre C, Quertermous T, Boss JM, Leiden JM, Seidman JG, Strominger JL: Cloning and sequence analysis of complementary DNA encoding an aberrantly rearranged human T-cell γ chain. Proc Natl Acad Sci USA 1986;83:2619-23.
51. Van den Elsen P, Shepley B-A, Borst J, Coligan JE, Markham AF, Orkin S, Terhorst C. Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex. Nature 1984;312:413-8.
52. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, Van de Griend RJ, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987; 17:1719-28.
53. Huck S, Lefranc M-P. Rearrangements to the JP1, JP and JP2 segments in the human T-cell rearranging gamma gene (TRGγ) locus. FEBS letters 1987;224:291-6.
54. Duby AD, Seidman JG. Abnormal recombination products result from aberrant DNA rearrangement of the human T-cell antigen receptor β- chain gene. Proc Natl Acad Sci USA 1986;83:4890-4.
55. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
56. Furley AJW, Chan LC, Mizutani S, Ford AM, Weilbaecher K, Pegram SM, Greaves MF. Lineage specificity of rearrangement and expression of genes encoding the T cell receptor-T3 complex and immunoglobulin heavy chain in leukemia. Leukemia 1987;1:644-52.
57. Iwashima M, Green A, Davis MM, Chien Y. Variable region (Vδ) gene segment most frequently utilized in adult thymocytes is 3′ of the constant (Cδ) region. Proc Natl Acad Sci USA 1988;85:8161-5.

58. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
59. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
60. Uppenkamp M, Pittaluga S, Lipford EH, Cossman J. Limited diversity and selection of rearranged γ genes in polyclonal T cells. J Immunol 1987;138:1618-20.
61. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc MP, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-83.
62. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain N segments and have marked junctional variability. Nature 1986;322:184-7.
63. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-4.
64. Koning F, Maloy WL, Cohen D, Coligan JE. Independent association of T cell receptor β and γ chains with CD3 in the same cell. J Exp Med 1987;166:595-600.
65. Laffan M, Foroni L, Rabbitts TH, Boehm T, Catovsky D, Luzzatto L. Rearrangement of the T-cell receptor delta (TCRδ) genes in human T cell leukaemias. Br J Haematol 1988;69:105.

CHAPTER 4.8

ANALYSIS OF PATIENTS WITH DIGEORGE ANOMALY PROVIDES EVIDENCE FOR EXTRATHYMIC DEVELOPMENT OF TcR-$\gamma\delta^+$ LYMPHOCYTES IN MAN*

Jacques J.M. van Dongen[1], W. Marieke Comans-Bitter[1,2], Wilhelm Friedrich[3], Herman J. Neijens[2], Bernd H. Belohradsky[4], Thomas Kohn[3,5], Anne Hagemeijer[6] and Jannie Borst[7]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands; 2. Department of Pediatrics, University Hospital/Sophia Children's Hospital, Rotterdam, The Netherlands; 3. Department of Pediatrics II, Universitäts-Kinderklinik Ulm, Ulm, FRG; 4. Abteilung für Antimikrobielle Therapie und Infektionsimmunologie, Kinderklinik der Universität im Dr. von Haunerschen Kinderspital, Münich, FRG; 5. Department of Pediatrics, Klinik Bayreuth, Bayreuth, FRG; 6. Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands; 7. Department of Immunology, Netherlands Cancer Institute, Amsterdam, The Netherlands.

SUMMARY

To investigate whether TcR-$\gamma\delta^+$ cells in man differentiate along a thymus-dependent pathway, we have analyzed the composition of T cells in peripheral blood of patients with DiGeorge Anomaly (DGA). DGA is an extremely rare, severe developmental field defect, that results in thymic hypoplasia or aplasia. The thymic defect is quantitative in that it solely involves a reduction of epithelial mass. The degree of T cell deficiency in DGA is related to the degree of thymus hypoplasia. In eight DGA patients, including one with a virtually complete absence of T lymphocytes (complete DGA), absolute numbers TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ cells in peripheral blood were determined. In some cases, also the repertoire of TcR-$\gamma\delta^+$ cells and their CD4/CD8 phenotype was analyzed with specific antibodies. Control values were collected by analyzing blood samples from a large number of healthy children in different age groups. Thymic hypoplasia or aplasia did not greatly affect the absolute numbers of TcR-$\gamma\delta^+$ cells, nor their repertoire and CD4/CD8 phenotype. In contrast, the development of TcR-$\alpha\beta^+$ cells was affected to a variable but significant degree, which in the complete DGA patient resulted in a complete inversion of the TcR-$\alpha\beta$/TcR-$\gamma\delta$ ratio (85% of the few remaining T cells expressed TcR-$\gamma\delta$). Also, thymic involution during maturation from infancy to adolescence coincided with a decrease in the absolute numbers of TcR-$\alpha\beta^+$ cells, but not TcR-$\gamma\delta^+$ cells, supporting the relationship between thymic cortical mass and the numbers of TcR-$\alpha\beta^+$ cells in blood. We conclude that TcR-$\gamma\delta^+$ cells in man can more effectively develop along an extrathymic differentiation pathway

* *Manuscript submitted.*

than TcR-$\alpha\beta^+$ cells and propose that human TcR-$\gamma\delta^+$ cells are subject to expansion and selection mechanisms in peripheral compartments.

INTRODUCTION

It has been accepted that mature TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ lymphocytes constitute distinct functional subsets, but their exact lineage interrelationship remains to be elucidated. It is unclear to what extent TcR-$\gamma\delta^+$ cells develop along the same thymus dependent differentiation pathway as TcR-$\alpha\beta^+$ cells and whether they undergo repertoire selection during this process. Evidence for extrathymic development of TcR-$\gamma\delta^+$ cells originally came from the observation that TcR-γ and TcR-δ messenger RNA was expressed in peripheral lymphoid organs of athymic (nude) mice (1,2). Recent studies in normal mice indicate that the TcR-$\gamma\delta^+$ cells present in murine epidermis originate from an early wave of fetal TcR-$\gamma\delta^+$ thymocytes at around day 15 of embryonic life, while the TcR-$\gamma\delta^+$ cells found in epithelia of the reproductive organs have emerged from the thymus just around the time of birth (3-9). These murine TcR-$\gamma\delta^+$ fetal thymocytes and their descendants in skin and reproductive organs have a homogeneous combinatorial repertoire of V5J1Cγ1/V1D2J2Cδ and V6J1Cγ1/V1D2J2Cδ gene segments, respectively (4-9). They have junctional regions with negligible nucleotide (N) insertion (5,6,9), which is in line with the absence in fetal thymocytes of the nuclear enzyme terminal deoxynucleotidyl transferase (TdT), that is thought to be responsible for N region insertion (10,11). These two thymus-dependent epithelial TcR-$\gamma\delta^+$ cell subsets with monomorphic receptors are indeed absent in nude mice (6,12), while such mice do contain TcR-$\gamma\delta^+$ cells in spleen (1,2,13), lymph nodes (14) and the intestinal epithelium (6). Apparently, the various TcR-$\gamma\delta^+$ cell populations in mice have different environmental requirements for development.

To study the thymus dependency of TcR-$\gamma\delta^+$ lymphocyte development in man, we have enumerated TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T cells in peripheral blood (PB) samples from eight infants with DiGeorge anomaly (DGA). DGA is characterized by facial, cardiac, parathyroid and thymic defects, due to malformations or disruptions of the third and fourth pharyngeal arches and pouches from which these organs develop (15-18). Furthermore, DGA is associated with chromosomal aberrations, particularly monosomy of chromosome 22 or deletion of chromosome band 22q11 (19).

The degree of thymus hypoplasia in DGA is variable; this defect involves thymic epithelial mass and is not a dysplastic process, i.e. it is a quantitative rather than a qualitative defect (16,18). It is generally assumed that the variable degree of T cell immunodeficiency in DGA is directly related to the degree of thymus hypoplasia or aplasia (16,18). DGA is an extremely rare and severe disorder. Complete DGA, characterized by total thymic aplasia and virtually complete absence of T cells, has according to a rough estimation an incidence of one in 10^6-10^7 newborns (16). One patient with complete DGA and seven patients with variable degrees of DGA have been included in our study.

TABLE 1. Characteristics of eight DGA patients.

Patients	K.H.	Y.G.	C.S.	B.D.	V.C.	M.V.	T.S.	A.L.
Age	3 mo	6 mo	1 mo	2 mo	2 mo	1 mo	7 mo	2 mo
DGA defects								
- facial dysmorphies	+	±	+	+	+	+	+	+
- cardiovascular abnormalities	+	+	+	+	+	+	+	+
- hypocalcemia	+	±	±	+	+	+	+	+
- absence of thymic shadow	+	+	+	+	+	+	+	+
Cytogenetics								
- deletion of band 22q11	+	NT	+	NT	NT	+	NT	+
Lymphocyte proliferation								
- PHA, ConA and CD3 (OKT3)	normal	normal	normal	↓	↓	↓	↓	absent

MATERIALS AND METHODS

DGA patients

PB samples of a total of eight DGA patients were collected at six different hospitals in Germany and The Netherlands, according to guidelines of the local Medical Ethics Committees. The DGA diagnosis of these patients was based on the presence of typical facial dysmorphies, cardio-vascular abnormalities, hypoparathyroidism with hypocalcemia, and/or the absence of a thymic shadow on thorax X-rays (16-18). At least three of these criteria had to be met for diagnosing DGA (Table 1). In addition, cytogenetic analysis of R-banded chromosome spreads from cultured PB cells was performed for four patients to evaluate the presence of constitutional aberrations of chromosome 22 (19). All four patients tested had a micro-deletion of band 22q11, supporting the DGA diagnosis. With respect to T cell immunodeficiency, the eight patients represented the broad spectrum from partial to complete DGA. As a hallmark of T cell immunodeficiency, the T cell proliferative response to mitogenic stimuli was measured. For this purpose, mononuclear cells (MNC) were incubated with phytohaemagglutinin (PHA), concanavalin A (Con A) and the CD3 antibody OKT3 (Table 1).

Healthy controls

To obtain normal values, PB samples were collected from ninety-two healthy children in different age-groups according to the guidelines of the Medical Ethics Committee of the Erasmus University/University Hospital Dijkzigt, Rotterdam. Upon routine physical examination no DGA defects were found. Serum calcium measurements and thorax X-rays were performed in only a minority of the children and found to be normal.

Monoclonal antibodies (McAb)

WT31 (20; Becton Dickinson, San Jose, CA) or BMA031 (21; Dr. R. Kurrle, Behringwerke AG, Marburg, FRG) were used to detect TcR-αβ, anti-TCR-γδ-1 (hybridoma 11F2, ref. 22, Dr. J. Borst, Amsterdam, The Netherlands) or TCRδ1 (23; T Cell Sciences, Cambridge, MA) to detect TcR-γδ and VIT-3 (Dr. W. Knapp, Vienna, Austria), Leu-4 (Becton Dickinson) or OKT3 (Ortho Diagnostic Systems, Raritan, NJ) to detect the CD3 antigen. The TcR-γδ

repertoire was analyzed with Vγ9-specific McAb Ti-γA (24; Dr. T. Hercend, Villejuif, France), Vδ2-specific McAb BB3 (25; Dr. L. Moretta, Genova, Italy) and Vδ1-Jδ1 specific McAb δTCS1 (26; T Cell Sciences). B cells were defined with CD20 McAb B1 (Coulter Clone, Hialeah, FL), monocytes with CD14 McAb My4 (Coulter Clone). The $CD4^+$ or $CD8^+$ phenotype of TcR-γδ cells was determined with Leu-3 and Leu-2 McAb respectively (Becton Dickinson).

Immunofluorescence (IF) procedures

MNC were isolated from PB by ficoll density centrifugation (density 1.077 g/ml). The composition of MNC from PB samples (Table 2) was determined by indirect IF staining, using unlabeled first McAb, followed by FITC-conjugated goat anti-mouse Ig (Nordic Immunological Laboratories, Tilburg, The Netherlands) as second step reagent. Staining was evaluated by flowcytometry with a FACScan (Becton Dickinson).

For double IF stainings presented in Figures 1 and 2, MNC were first incubated with one of the anti-TcR McAb (all of the murine IgG class) and with VIT-3 (IgM class), followed by incubation with FITC-conjugated goat anti-mouse IgG and TRITC-conjugated goat anti-mouse IgM (Nordic) to detect the reactivity of the anti-TcR and the CD3 McAb, respectively. After labeling, cytocentrifuge preparations were made (Cytofuge, Nordic) and analyzed with a fluorescence microscope (Zeiss, Oberkochen, FRG) (27) (Figure 1). In each double IF staining, at least 500 $CD3^+$ cells were evaluated for TcR-αβ or TcR-γδ expression. In all MNC samples of Figure 2, the expression of TcR-αβ was determined with WT31 as well as BMA031 McAb, while the expression of TcR-γδ was determined with anti-TCR-γδ-1 as well as TCRδ1 McAb. In both cases, the numbers obtained with each McAb were virtually identical (<2% discrepancy).

For double IF stainings presented in Table 2, MNC were first incubated with Ti-γA, δTCS1, BB3, Leu-3 or Leu-2 McAb, followed by TRITC-conjugated goat anti-mouse Ig (Nordic Immunological Laboratories). Subsequently, free antigen binding sites of the second step reagent were blocked by incubation with normal mouse serum (dilution 1:100), followed by biotinylated anti-TCR-γδ-1 and FITC-conjugated streptavidin (Boehringer Mannheim, Mannheim, FRG). Cytocentrifuge preparations were made and evaluated by fluorescence microscopy as outlined in Chapter 2.3. If available, at least 200 TcR-$\gamma\delta^+$ cells were evaluated for reactivity with Ti-γA, δTCS1, BB3, Leu-3 or Leu-2.

RESULTS

PB samples from eight DGA patients were collected over a period of two years and analyzed in this study in order to investigate the thymus dependency of TcR-$\gamma\delta^+$ cell differentiation in man. The patients varied in age from one to seven months. Their characteristics, with respect to the various clinical manifestations of the disease, are given in Table 1. Patient A.L. was diagnosed as having complete DGA.

It was determined to what extent the T lymphocyte population was decreased in the various DGA patients. T cells, B cells and monocytes were enumerated by IF staining, using CD3, CD20 and CD14 McAb, respectively (Table 2). We are aware that the cumulative percentages of B cells, T cells and monocytes do not add up to 100%. This is due to the presence of variable percentages of NK cells and immature granulocytes (rods), which can accumulate in PB of patients with recurrent infections. The absolute numbers of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ cells were determined in order to investigate the effect of the thymic defect on both T cell lineages. MNC were analyzed by double IF stainings with CD3 and anti-TcR McAb, in order to measure directly the relative distribution of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ cells within the $CD3^+$ lymphocyte population. Stained cells were analyzed by fluorescence microscopy, rather than flowcytometry, because of its high accuracy, needed when very small cell populations (<1% of MNC) are studied. To obtain control values, the same analysis was performed on MNC from a large num-

TABLE 2. Composition of MNC population in DGA patients and healthy age-matched controls.

	DGA patients								Healthy children
	K.H.	Y.G.	C.S.	B.D.	V.C.	M.V.	T.S.	A.L.	(n=17)
Age	3 mo	6 mo	1 mo	2 mo	2 mo	1 mo	7 mo	2 mo	0-6 mo
Total T cells(CD3⁺)									
% of MNC	44	36	42	22	24	25	8.0	1.1	59 ± 11[b]
# x 10^9/l[a]	2.5	2.5	1.9	1.4	1.0	0.8	0.14	0.03	3.9 ± 1.4
TcR-αβ⁺ cells									
% of MNC	41	36	39	20	22	25	7.0	0.04	55 ± 10
# x 10^9/l	2.4	2.4	1.7	1.3	0.96	0.8	0.12	0.002	3.6 ± 1.3
TcR-γδ⁺ cells									
% of MNC	2.2	1.8	2.5	1.3	1.1	1.0	1.1	0.9	2.6 ± 1.5
# x 10^9/l	0.12	0.10	0.11	0.08	0.05	0.02	0.02	0.03	0.16 ± 0.09
B cells(CD20⁺)									
% of MNC	24	18	10	12	23	8	31	44	15 ± 8
# x 10^9/l	1.4	1.3	0.5	0.8	1.0	0.3	0.6	1.3	1.0 ± 6
Monocytes(CD14⁺)									
% of MNC	14	24	24	11	22	21	37	38	15 ± 9
# x 10^9/l	0.8	1.7	1.1	0.7	1.0	0.7	0.7	1.1	1.0 ± 0.6

a. Absolute numbers x 10^9 per liter blood.
b. Mean ± standard deviation.

ber of healthy children (n=92) in five different age groups, including seventeen children in the age group of 0-6 months, which represents the age-matched control group for the DGA patients.

Table 2 illustrates that the absolute number of T cells is normal or reduced to some degree in six DGA patients (K.H.-M.V.). In case of reduction, the absolute number of TcR-αβ⁺ cells is reduced relatively more than the absolute number of TcR-γδ⁺ cells. But these differences are limited in patients K.H.-M.V. However, in patient T.S. and particularly in the complete DGA patient A.L., the absolute number of T cells has dropped dramatically. TcR-γδ⁺ cells are affected in numbers to a factor of about three or four below normal age matched control values, but strikingly, TcR-αβ⁺ cells are affected to a factor of about 20 in patient T.S. and even a factor 1000 in patient A.L.

A graphic representation of these data is given in Figure 2. Figure 2A shows the absolute numbers of total T cells, subdivided in TcR-αβ⁺ and TcR-γδ⁺ populations for all DGA patients, and in addition, for healthy children of the five different age groups from 0-16 years old. As indicated in Table 2, the reference for DGA patients is the age group of 0-6 months. Figure 2B gives the relative contribution of TcR-αβ⁺ and TcR-γδ⁺ cells to the total CD3⁺ population for

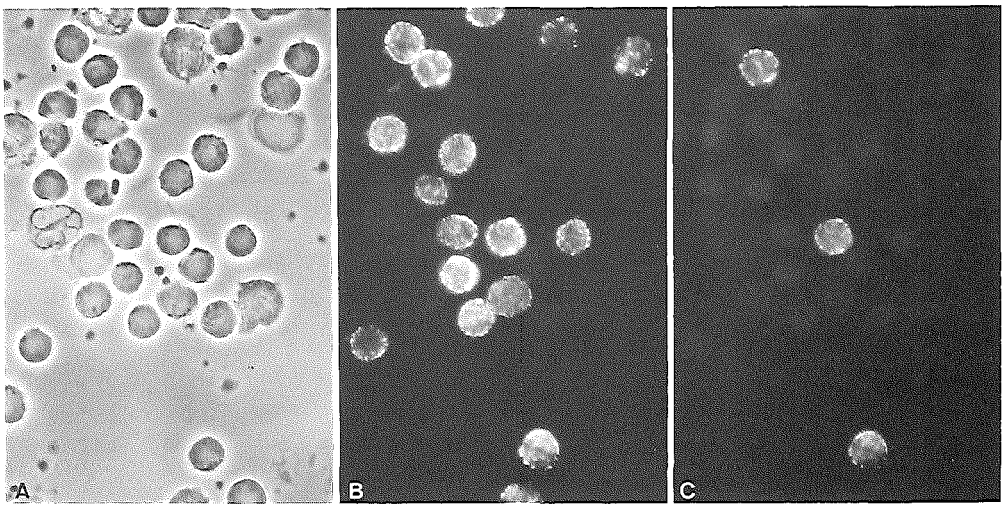


Figure 1. Analysis of MNC from PB of a healthy individual for coexpression of CD3 and TcR-$\gamma\delta$ by fluorescence microscopy. **A**: Phase contrast morphology; **B**: $CD3^+$ cells stained with VIT-3 and a TRITC-conjugated second step reagent; **C**: TcR-$\gamma\delta^+$ cells stained with anti-TCR-$\gamma\delta$-1 and a FITC conjugated second step reagent. All three photographs represent the same microscopic field: three $CD3^+$ cells are also positive for TcR-$\gamma\delta$. Methods are outlined in the Materials and Methods section.

the DGA patients and the same groups of healthy children, as determined by double IF stainings with CD3 and anti-TcR McAb. This graph clearly illustrates how in the six patients K.H.-M.V., the percentage of TcR-$\gamma\delta^+$ cells is normal or only slightly higher than in controls, while in patient T.S. and particularly in patient A.L. the severe reduction in absolute numbers of TcR-$\alpha\beta^+$ cells relative to TcR-$\gamma\delta^+$ cells has resulted in a significant increase in the percentage of TcR-$\gamma\delta^+$ cells. In patient A.L., 85% of the few remaining $CD3^+$ cells express TcR-$\gamma\delta$, while only 5% of the $CD3^+$ cells reacted with anti-TcR-$\alpha\beta$ McAb.

It is remarkable that in healthy children the absolute numbers of T cells gradually decrease from about 4×10^9/l in early infancy to approximately 1.5×10^9/l in adolescence (Figure 2A). This decrease only concerns TcR-$\alpha\beta^+$ cells, since the absolute numbers of TcR-$\gamma\delta^+$ cells remain stable (Figure 2A). This is also reflected in the increase of the percentage TcR-$\gamma\delta^+$ cells within the $CD3^+$ population with age (Figure 2B).

Since no data are available on thymic repertoire selection of TcR-$\gamma\delta^+$ cells in man, it was of interest to analyze the DGA patients in this respect. Three McAb are available, Ti-γA, δTCS1 and BB3, that detect epitopes encoded by Vγ9, Vδ1-Jδ1 and Vδ2 gene segments, respectively (24-26,28) and are therefore useful to analyze the TcR-$\gamma\delta^+$ cell repertoire. It has previously been established that the combinatorial repertoire of TcR-$\gamma\delta^+$ in PB of healthy adults is very limited, with the Vγ9/Vδ2 combination being expressed on >60% of TcR-$\gamma\delta^+$ cells in most individuals (24,28). The TcR-$\gamma\delta^+$ cell populations in PB of ten healthy children, two incomplete DGA patients and the one patient with complete DGA were analyzed (Table 3). Despite some individual variability, the relative distribution of Vγ and Vδ gene segment usage did not differ greatly between these samples. The majority of TcR-$\gamma\delta^+$ cells were positive for Vγ9 (Ti-γA) and/or Vδ2

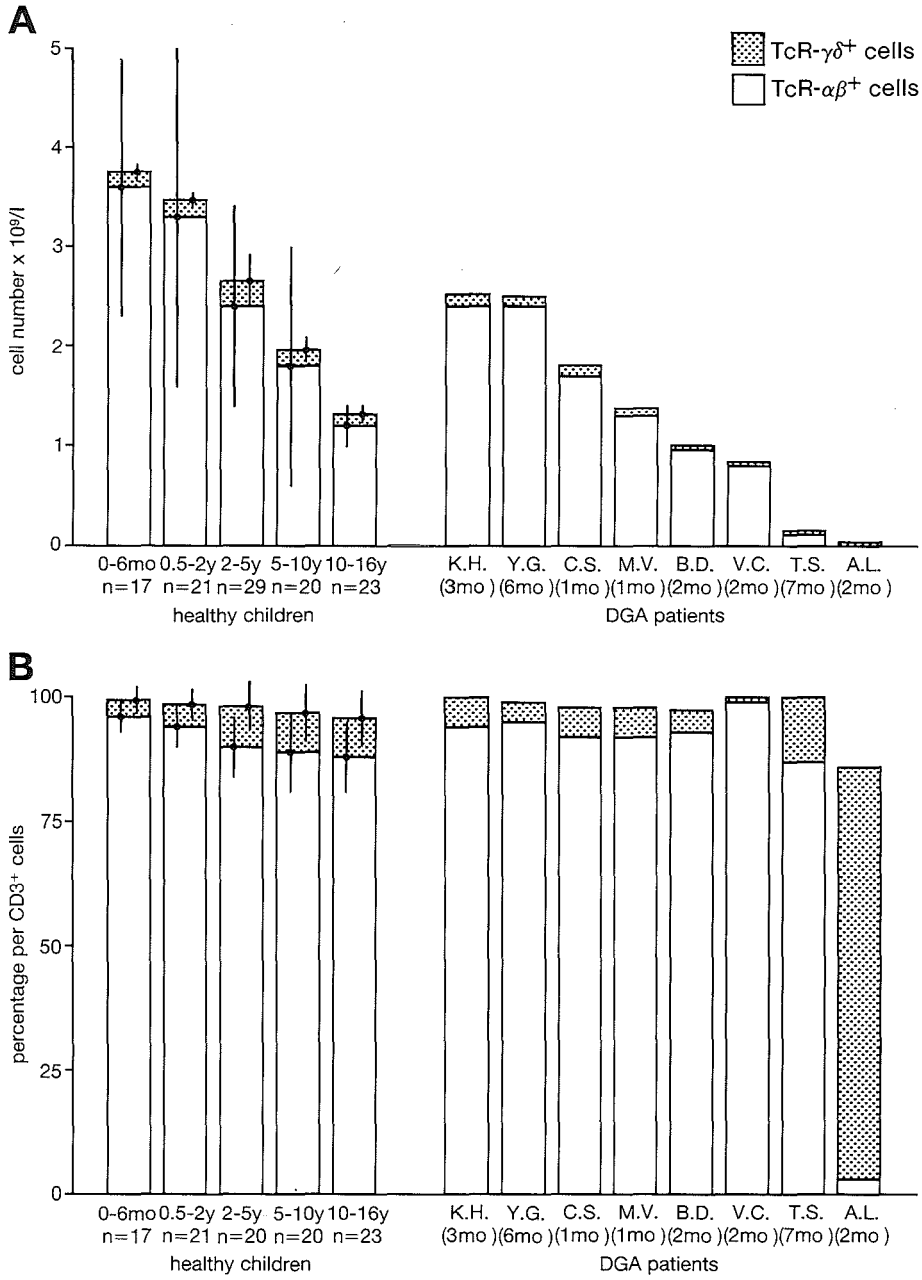


Figure 2. Expression of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ by $CD3^+$ lymphocytes in PB of DGA patients and healthy children of three different age-groups. **A**: Absolute numbers of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T cells. **B**: Relative distribution of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ expression within the population of $CD3^+$ T cells. Double IF was performed as indicated in the Materials and Methods section. In patient T.S. only single IF stainings were used; the percentages per $CD3^+$ cells in this patient were calculated from the single IF stainings. For healthy children the mean ± the standard deviation (vertical lines within the bars) is indicated.

(BB3), while a small proportion expressed Vδ1-Jδ1 (δTCS1). This was found in all patients, including complete DGA patient A.L., as well as in healthy controls.

Since one could imagine that TcR-$\gamma\delta^+$ cells, like TcR-$\alpha\beta^+$ cells, would pass through a $CD4^+/CD8^+$ double positive stage during thymic differentiation in order to acquire the single positive $CD4^+$ or $CD8^+$ phenotype (29), the expression of these accessory molecules was determined in selected patient materials and in healthy controls (Table 3). Although the majority of TcR-$\gamma\delta^+$ cells was found to have the $CD4^-/CD8^-$ phenotype (in contrast to TcR-$\alpha\beta^+$ cells), a low, but detectable percentage of TcR-$\gamma\delta^+$ cells in healthy persons expressed CD4, while a significant proportion was $CD8^+$, with great variation between individuals (see also refs. 22 and 28). It can be seen in Table 3, that in DGA patients the percentages of $CD4^+$ and $CD8^+$ TcR-$\gamma\delta^+$ cells did not differ significantly from those in healthy individuals. Strikingly, also in the complete DGA patient A.L. 13% of TcR-$\gamma\delta^+$ cells expressed CD8. Apparently, acquisition of this phenotype is not exclusively thymus dependent.

DISCUSSION

Our combined data indicate that in DGA patients, despite hypoplasia or aplasia of the thymus, the absolute numbers of TcR-$\gamma\delta^+$ cells as well as their combinatorial repertoire and CD4/CD8 phenotype are virtually comparable to those in age-matched healthy children. In contrast, there is a severe effect on TcR-$\alpha\beta^+$ cell numbers. It is generally assumed that the thymic defect in DGA is a quantitative reduction in thymic epithelial mass (16,18). Therefore, we conclude that TcR-$\alpha\beta^+$ cell development in man is severely hampered by the decreased availability of thymic epithelial environment, while TcR-$\gamma\delta^+$ cell development is much less affected. This suggests that TcR-$\gamma\delta^+$ cells can differentiate along an extrathymic pathway. These findings in man are comparable to those documented for the athymic (nude) mouse (1,2). Interestingly, we found that in healthy children the absolute number of TcR-$\alpha\beta^+$ cells gradually decreases approximately three-fold during maturation from infancy to adolescence, while the absolute number of TcR-$\gamma\delta^+$ cells remains stable during this period. Simultaneously a prominent involution of the thymic cortex occurs (30). This coincidence of thymic involution and selective decrease of TcR-$\alpha\beta^+$ cells is in line with the relationship between thymic mass and absolute numbers of TcR-$\alpha\beta^+$ cells as found in DGA patients.

So far it is unclear to what extent the human thymus contributes to the maturation of TcR-$\gamma\delta^+$ cells. Unlike the situation in the mouse, TcR-$\gamma\delta^+$ cells in man do not predominate in the fetal thymus at any time-point (31). There seems to be no equivalent in man for the two thymus dependent TcR-$\gamma\delta^+$ cell populations found in epidermis and epithelia of reproductive organs of mice, since they are the far minority of T cells at these sites (32-34). TcR-$\gamma\delta^+$ cells are present in the human thymus (22), but as a very low percentage of $CD3^+$ cells (<1%) (22,35) and occur in higher frequencies in the medulla than in the cortex (32,36). $CD3^-/CD4^-/CD8^-$ prothymocytes can gives rise to TcR-$\gamma\delta^+$ cells *in vitro* (37). However, because of obvious experimental constraints, no direct precursor-product relationships between prothymocytes and mature TcR-$\gamma\delta^+$ cells in PB have been determined *in vivo*. The here observed three to four fold reduction in absolute number of TcR-$\gamma\delta^+$ cells in DGA patients might suggest that the thymus does contribute to the development of TcR-$\gamma\delta^+$ cells, but less than for TcR-$\alpha\beta^+$ cells.

TABLE 3. TcR-γδ repertoire and CD4/CD8 expression in PB of DGA patients and healthy children.

	Partial DGA patients		Complete DGA patient	Healthy children
	B.D.	V.C.	A.L.	n=10
Age	2 mo	2 mo	2 mo	2 mo - 2 y
Vγ 9(Ti-γA)⁺/TcR-γδ⁺[a]	95	92	80	72 ± 19[b] (32-91)[c]
Vδ1-Jδ1 (δTCS1)⁺/TcR-γδ⁺	1	1	1	20 ± 14 (5-46)
Vδ2(BB3)⁺/TcR-γδ⁺	NT	NT	79	77 ± 18 (38-92)
CD4(Leu-3)⁺/TcR-γδ⁺	8	0	1	2 ± 1 (0-4)
CD8(Leu-2)⁺/TcR-γδ⁺	45	13	13	17 ± 5 (9-30)

a. Percentage positivity within TcR-γδ⁺ population.
b. Mean ± standard deviation.
c. Range.

Our data derived from the complete DGA patient A.L. suggest that TcR-γδ⁺ cells can expand extra-thymically into a population that is only three to four fold smaller in absolute numbers than in age-matched healthy children, while the TcR-αβ⁺ cell population remains 1000-fold smaller than in healthy children. Also, as in many healthy individuals, the peripheral repertoire of the complete DGA patient A.L. is dominated by the Vγ9⁺/Vδ2⁺ populations (24,28). Apparently, the combinatorial repertoire of human TcR-γδ⁺ cells is regulated to a large extent by selection mechanisms in the periphery. We postulate that the Vγ9⁺/Vδ2⁺ subsets of TcR-γδ⁺ cells is particularly prone to positive selection and expansion in the periphery. The activating and positively selecting element can either be a self MHC (-like?) molecule, or an ubiquitous antigen that would have to be categorized as a superantigen given its effect on such a large, but defined subset of TcR-γδ⁺ cells.

ACKNOWLEDGMENTS. We thank Prof. Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Dr. R. de Groot for collecting blood samples of healthy children, Dr. R.N. Sukhai, Prof. Dr. G. Wündisch, Dr. M. Kellner, Prof. dr. P. Sachtleben, Dr. B. Müller, Dr. D. Vögtel, and Prof. Dr. J.G. Schröber for kindly providing PB samples from DGA patients, Dr. R. Kurrle, Dr. T. Hercend, Dr. L. Moretta, and Dr. W. Knapp for providing antibodies, Mr. E. de Vries for technical assistance, Mr. T.M. van Os for his contribution to preparing the figures and Ms. E.P. Schenkel for secretarial help.

This work was supported by the Netherlands Organization for Scientific Research (J. Borst, grant H93-155).

REFERENCES

1. Yoshikai Y, Reis MD, Mak TW. Athymic mice express a high level of functional γ-chain but greatly reduced levels of α- and β-chain T-cell receptor messages. Nature 1986;324:482-5.
2. Yoshikai Y, Matsuzaki G, Takeda Y, Ohga S, Kishihara K, Yuuki H, Nomoto K. Functional T cell receptor δ chain gene messages in athymic nude mice. Eur J Immunol 1988;18:1039-43.

3. Havran WL, Allison JP. Developmentally ordered appearance of thymocytes expressing different T-cell antigen receptors. Nature 1988;335:443-5.
4. Havran WL, Grell S, Duwe G, Kimura J, Wilson A, Kruisbeek AM, O'Brien RL, Born W, Tigelaar RE, Allison JP. Limited diversity of T-cell receptor γ-chain expression of murine Thy-1$^+$ dendritic epidermal cells revealed by Vγ3-specific monoclonal antibody. Proc Natl Acad Sci USA 1989;86:4185-9.
5. Lafaille JJ, DeCloux A, Bonneville M, Takagaki Y, Tonegawa S. Junctional sequences of T cell receptor γδ genes: implications for γδ T cell lineages and for a novel intermediate of V-(D)-J joining. Cell 1989;59:859-70.
6. Tonegawa S, Berns A, Bonneville M, Farr AG, Ishida I, Ito K, Itohara S, Janeway CA, Kanagawa O, Katsuiki M, Kubo R, Lafaille JJ, Mombaerts P, Murphy DB, Nakanishi N, Takagaki Y, Van Kaer L, Verbeek S. Diversity, development, ligands and probable functions of γδ T cells. Progr Immunol 1989;7:243-57.
7. Ito K, Bonneville M, Takagaki Y, Nakanishi N, Kanagawa O, Krecko EG, Tonegawa S. Different γδ T-cell receptors are expressed on thymocytes at different stages of development. Proc Natl Acad Sci USA 1989;86:631-5.
8. Itohara S, Nakanishi N, Kanagawa O, Kubo R, Tonegawa S. Monoclonal antibodies specific to native murine T-cell receptor γδ: analysis of γδ T cells during thymic ontogeny and in peripheral lymphoid organs. Proc Natl Acad Sci USA 1989;86:5094-8.
9. Asarnow DM, Goodman T, LeFrancois L, Allison JP. Distinct antigen repertoires of two classes of murine epithelium-associated T cells. Nature 1989;341:60-2.
10. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-J_H fusions. Proc Natl Acad Sci USA 1982;79:4118-22.
11. Rothenberg E, Triglia D. Clonal proliferation unlinked to terminal deoxynucleotidyl transferase synthesis in thymocytes of young mice. J Immunol 1983;130:1627-33.
12. Nixon-Fulton JL, Kuziel WA, Santerse B, Bergstresser PR, Tucker PW, Tigelaar RE. Thy-1$^+$ epidermal cells in nude mice are distinct from their counterparts in thymus-bearing mice. J Immunol 1988;141:1897-3.
13. Pardoll DM, Fowlkes BJ, Lew AM, Maloy WL, Weston MA, Bluestone JA, Schwartz RH, Coligan JE, Kruisbeek AM. Thymus-dependent and thymus-independent developmental pathways for peripheral T cell receptor-γδ-bearing lymphocytes. J Immunol 1988;140:4091-6.
14. Matis LA, Cron R, Bluestone JA. Major histocompatibility complex-linked specificity of γδ receptor-bearing T lymphocytes. Nature 1987;330:262-4.
15. DiGeorge AM. Congenital absence of the thymus and its immunologic consequences: concurrence with congenital hypoparathyroidism. Birth Defects 1986;4:116-21.
16. Belohradsky BH. Thymusaplasie und -hypoplasie mit Hypoparathyreo-idismus, Herz-und Gefässmissbildungen (Di-George-Syndrom). Ergebnisse der Inneren Medizin und Kinderheilkunde 1985;54:35-105.
17. Lammer EJ, Opitz JM. The DiGeorge Anomaly as a developmental field defect. Am J Med Genet Supplement 1986;2:113-27.
18. Bastian J, Law S, Vogler L, Lawton A, Herrod H, Anderson S, Horowitz S, Hong R. Prediction of persistent immunodeficiency in the DiGeorge anomaly. J Pediatr 1989;15:391-6.
19. Greenberg F, Elder FFB, Haffner P, Northrup H, Ledbetter DH. Cytogenetic findings in a prospective series of patients with DiGeorge anomaly. Am J Hum Genet 1988;43:605-11.
20. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 135:1922-8.
21. Borst J, Van Dongen JJM, De Vries E, Comans-Bitter WM, Van Tol MJD, Vossen JM, Kurrle R. BMA031, a monoclonal antibody suited to identify the T cell receptor αβ/CD3 complex on viable human T lymphocytes in normal and disease states. Hum Immunol, in press.
22. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor γ/δ detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.
23. Band H, Hochstenbach F, McLean J, Hata S, Krangel MS, Brenner MB. Immunochemical proof that a novel rearranging gene encodes the T cell receptor δ subunit. Science 1987;238:682-4.
24. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-8.

25. Ciccone E, Ferrini S, Bottino C, Viale O, Progione I, Pantaleo G, Tambussi G, Moretta A, Moretta L. A monoclonal antibody specific for a common determinant of the human T cell receptor γ/δ directly activates $CD3^+WT31^-$ lymphocytes to express their functional program(s). J Exp Med 1988;168:1-11.
26. Wu Y-J, Tian W-T, Snider RM, Rittershaus C, Rogers P, LaManna L, Ip SH. Signal transduction of γ/δ T cell antigen receptor with a novel mitogenic anti-δ antibody. J Immunol 1988;141:1476-9.
27. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.
28. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.
29. Von Boehmer H, Kisielow P, Kishi H, Scott B, Borgulya P, Teh HS. The expression of CD4 and CD8 accessory molecules on mature T cells is not random but correlates with the specificity of the $\alpha\beta$ receptor for antigen. Immunol Rev 1989;109:143-51.
30. Steinmann GG, Klaus B, Müller-Hermelink H-K. The involution of the aging human thymic epithelium is independent of puberty. Scand J Immunol 1985;22:563-75.
31. Campana D, Janossy G, Coustan-Smith E, Amlot PL, Tian W-T, Ip S, Wong L. The expression of T cell receptor-associated proteins during T cell ontogeny in man. J Immunol 1989;142:57-66.
32. Groh V, Porcelli S, Fabbi M, Lanier LL, Picker LJ, Anderson T, Warnke RA, Bhan AK, Strominger JL, Brenner MB. Human lymphocytes bearing T cell receptor γ/δ are phenotypically diverse and evenly distributed throughout the lymphoid system. J Exp Med 1989;169:1277-94.
33. Vroom TM, Bos JD, Borst J. Epithelial homing of $\gamma\delta$ T cells. Nature 1989;341:114.
34. Bos JD, Teunissen MBM, Cairo I, Krieg SR, Kapsenberg ML, Das PK, Borst J. T-cell receptor $\gamma\delta$ bearing cells in normal human skin. J Invest Dermatol 1990;94:37-42.
35. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus dependency. Thymus 1990, in press.
36. Falini B, Flenghi L, Pileri S, Pelicci P, Fagioli M, Martelli MF, Moretta L, Ciccone E. Distribution of T cells bearing different forms of the T cell receptor γ/δ in normal and pathological human tissues. J Immunol 1989;143:2480-8.
37. Toribio ML, De la Hera A, Borst J, Marcos MAR, Márquez C, Alonso JM, Bárcena A, Martínez-AC. Involvement of the interleukin 2 pathway in the rearrangement and expression of both α/β and γ/δ T cell receptor genes in human T cell precursors. J Exp Med 1988;168:2231-49.

CHAPTER 5

CLINICAL APPLICATIONS OF IMMUNOLOGIC MARKER ANALYSIS

CHAPTER 5.1

INTRODUCTION: IMMUNOPHENOTYPING OF LEUKEMIAS AND NON-HODGKIN LYMPHOMAS*

J.J.M. van Dongen, H.J. Adriaansen and H. Hooijkaas

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

The different types of hematologic malignancies can be regarded as malignant counterparts of cells in the various stages of hematopoiesis (1,2). Differentiation schemes such as presented in Figures 1 and 2 are based on data concerning normal hematopoiesis as well as on data concerning leukemias and non-Hodgkin lymphomas (NHL) (1,2). The lymphoid differentiation consists of B cell and T cell lineages, while the myeloid differentiation consists of monocytic, granulocytic, erythroid and thrombocytic lineages.

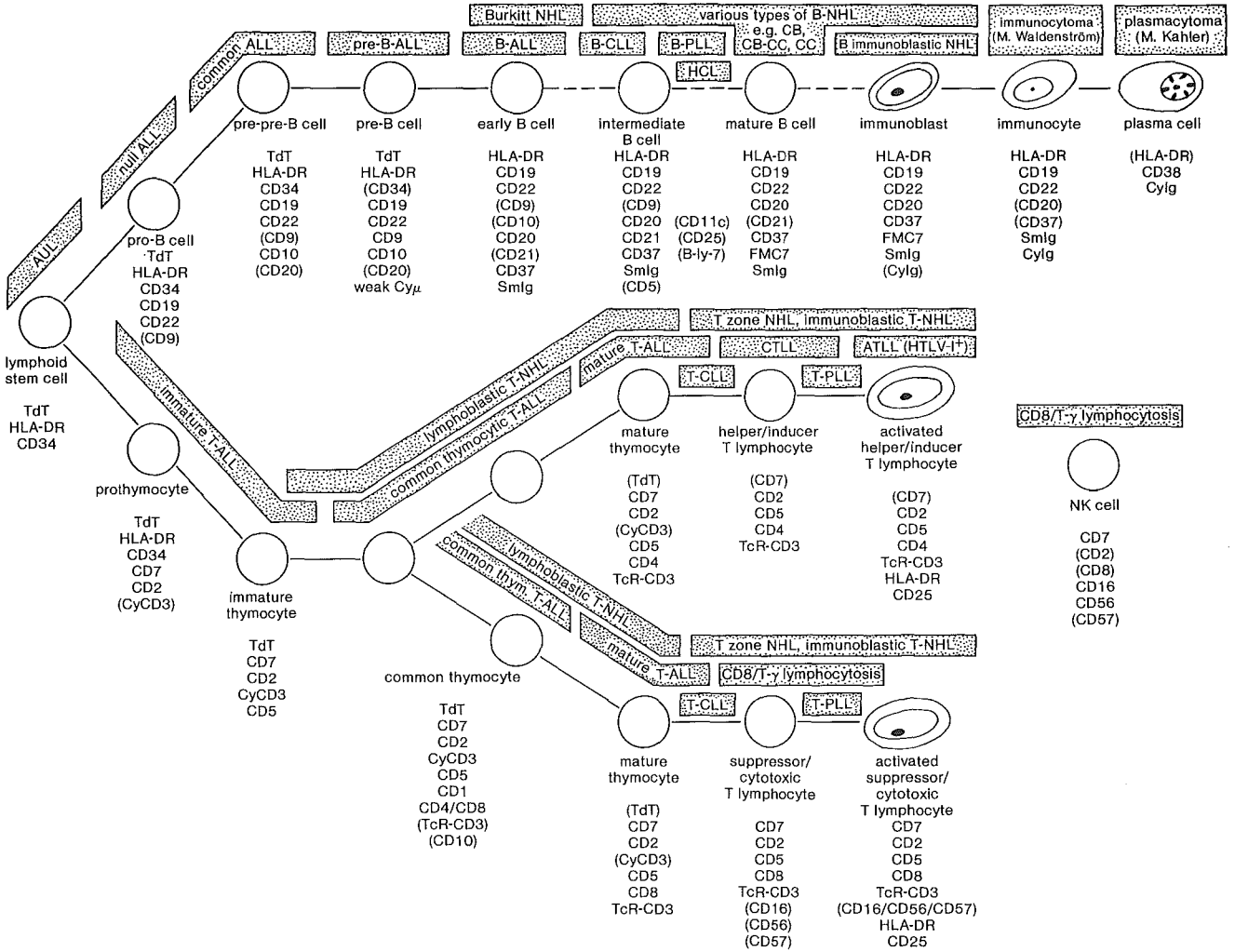
The majority of the cells in the various differentiation stages and their malignant counterparts can be recognized morphologically. However, immunologic marker analysis allows a further characterization of these cells. Immunologic markers are (glyco)proteins, which are located on the cell surface or intracellularly. These proteins are called immunologic markers, because they can be detected by means of immunologic techniques, in which the protein is generally recognized by use of a specific antibody.

We will discuss several immunologic markers, the recently developed nomenclature for antibodies against immunologic markers and the expression of immunologic markers during hematopoietic differentiation. Subsequently we will indicate the possibilities of immunologic marker analysis for diagnosis and management of leukemias and NHL.

IMMUNOLOGIC MARKERS AND THE CD NOMENCLATURE

For the detection of immunologic markers predominantly monoclonal antibodies (McAb) are used. During the last 10 years many laboratories have produced McAb against the various immunologic markers. To create order and clearness within the large pool of these antibodies, a special nomenclature has been developed during four Leucocyte Typing Conferences (Paris 1982; Boston 1984; Oxford 1986; Vienna 1989). According to this internationally accepted no-

Published in: Neth J Med 1988;33:298-314. Updated for CD codes and several new immunologic markers. The updated Appendix of this manuscript is included in Chapter 2.2.


Burkitt NHL
various types of B-NHL
e.g. CB, CB-CC, CC
immunocytoma (M. Waldenström)
plasmacytoma (M. Kahler)
common ALL
pre-B-ALL
B-ALL
B-CLL
B-PLL
HCL
B immunoblastic NHL
null ALL
AUL
pre-pre-B cell
pre-B cell
early B cell
intermediate B cell
mature B cell
immunoblast
immunocyte
plasma cell
TdT HLA-DR CD34 CD19 CD22 (CD9) CD10 (CD20)
TdT HLA-DR (CD34) CD19 CD22 CD9 CD10 (CD20) weak Cyμ
HLA-DR CD19 CD22 (CD9) (CD10) CD20 (CD21) CD37 SmIg
HLA-DR CD19 CD22 (CD9) CD20 (CD11c) CD21 (CD25) CD37 (B-ly-7) SmIg (CD5)
HLA-DR CD19 CD22 CD20 (CD21) CD37 FMC7 SmIg
HLA-DR CD19 CD22 CD20 CD37 FMC7 SmIg (CyIg)
HLA-DR CD19 CD22 (CD20) (CD37) SmIg CyIg
(HLA-DR) CD38 CyIg
pro-B cell TdT HLA-DR CD34 CD19 CD22 (CD9)
lymphoid stem cell TdT HLA-DR CD34
T zone NHL, immunoblastic T-NHL
lymphoblastic T-NHL
mature T-ALL
CTLL
ATLL (HTLV-I+)
immature T-ALL
common thymocytic T-ALL
T-CLL
T-PLL
mature thymocyte (TdT) CD7 CD2 (CyCD3) CD5 CD4 TcR-CD3
helper/inducer T lymphocyte (CD7) CD2 CD5 CD4 TcR-CD3
activated helper/inducer T lymphocyte (CD7) CD2 CD5 CD4 TcR-CD3 HLA-DR CD25
CD8/T-γ lymphocytosis
NK cell CD7 (CD2) (CD8) CD16 CD56 (CD57)
prothymocyte TdT HLA-DR CD34 CD7 CD2 (CyCD3)
immature thymocyte TdT CD7 CD2 CyCD3 CD5
common thymocyte TdT CD7 CD2 CyCD3 CD5 CD1 CD4/CD8 (TcR-CD3) (CD10)
lymphoblastic T-NHL
common thym. T-ALL
mature T-ALL
T zone NHL, immunoblastic T-NHL
CD8/T-γ lymphocytosis
T-CLL
T-PLL
mature thymocyte (TdT) CD7 CD2 (CyCD3) CD5 CD8 TcR-CD3
suppressor/ cytotoxic T lymphocyte CD7 CD2 CD5 CD8 TcR-CD3 (CD16) (CD56) (CD57)
activated suppressor/ cytotoxic T lymphocyte CD7 CD2 CD5 CD8 TcR-CD3 (CD16/CD56/CD57) HLA-DR CD25

menclature many McAb against immunologic markers have received their own code: the CD (= cluster of differentiation/cluster designation) code (3-6). For example, the McAb against T cell markers T4 and T8 have received the codes CD4 and CD8, respectively, while antibodies against the common acute lymphoblastic leukemia antigen (CALLA) are grouped into the CD10 cluster.

Behind the CD code one can place within parentheses the name of the McAb, which is used to detect the marker concerned. In this way, code CD10 can be extended to CD10(J5), CD10(BA-3) or CD10(VIL-A1), depending on the antibody used. Such a supplementation can be important for optimal comparison of data from different laboratories, since some McAb that recognize the same marker may slightly differ in their reaction pattern.

Information about all clustered immunologic markers and several relevant non-clustered markers is summarized in the Appendix of Chapter 2.2.

EXPRESSION OF IMMUNOLOGIC MARKERS DURING HEMATOPOIESIS

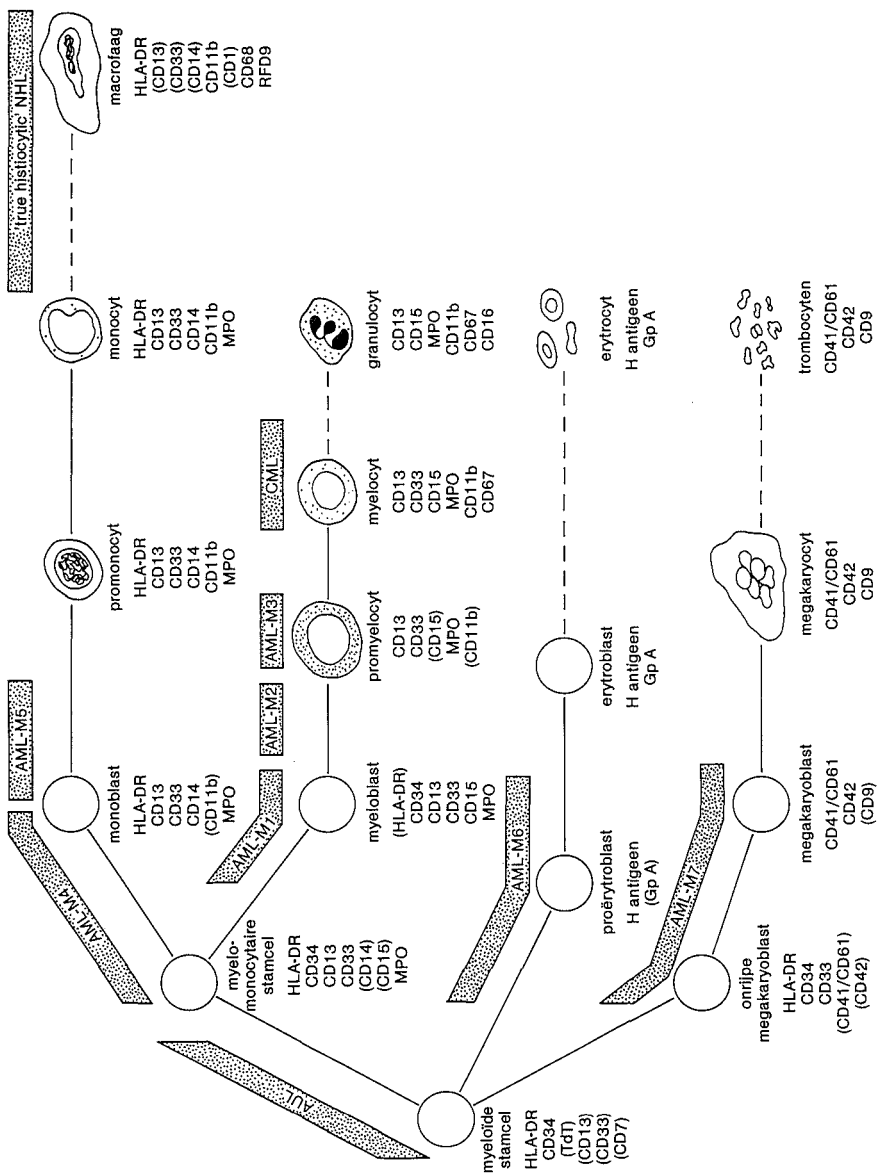
The majority of the immunologic markers represent differentiation antigens. Their expression is generally not restricted to a single differentiation stage, but occurs in several differentiation stages, sometimes even in several differentiation lineages. However, a combination of markers can be specific for one particular differentiation stage. In Figures 1 and 2 we have tried to indicate for each differentiation stage the expression of the most relevant immunologic markers. These markers, their CD codes and their alternative names are summarized in Table 1.

Lymphoid markers

The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed on the nuclear membrane of immature lymphoid cells, but is absent in more mature lymphoid differentiation stages (7). This expression pattern is related to the important role of TdT in the rearrangement of immunoglobulin (Ig) and T cell receptor (TcR) genes during the early stages of lymphopoiesis (8,9).

The B cell markers CD19 and CD22 are expressed at the cell surface in virtually all stages of B cell differentiation (10-12). Other B cell markers (such as CD10, CD20, CD21, CD37, FMC7 and B-ly-7) as well as the various forms of Ig expression can be used for further characterization of cells during B cell differentiation (10,11,13). The pre-B cell stage, for example, is char-

Figure 1. Hypothetical scheme of lymphoid differentiation. The expression of relevant immunologic markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and NHL and indicate where these malignancies can be located according to their maturation arrest: ALL, acute lymphoblastic leukemia; ATLL, adult T cell leukemia lymphoma; AUL, acute undifferentiated leukemia; CB, centroblastic lymphoma; CB-CC, centroblastic-centrocytic lymphoma; CC, centrocytic lymphoma; CLL, chronic lymphocytic leukemia; CTLL, cutaneous T cell leukemia lymphoma (mycosis fungoides/Sézary syndrome); HCL, hairy cell leukemia; HTLV, human T cell leukemia virus; NHL, non-Hodgkin lymphoma; PLL, prolymphocytic leukemia.


AML-M5
AML-M4
AML-M1
AML-M2
AML-M3
CML
AML-M6
AML-M7
AUL
'true histiocytic' NHL
myelo-monocytaire stamcel
HLA-DR CD34 CD13 CD33 (CD14) (CD15) MPO
monoblast
HLA-DR CD13 CD33 CD14 (CD11b) MPO
promonocyt
HLA-DR CD13 CD33 CD14 CD11b MPO
monocyt
HLA-DR CD13 CD33 CD14 CD11b MPO
macrofaag
HLA-DR (CD13) (CD33) (CD14) CD11b (CD1) CD68 RFD9
myeloblast
(HLA-DR) CD34 CD13 CD33 CD15 MPO
promyelocyt
CD13 CD33 (CD15) MPO (CD11b)
myelocyt
CD13 CD33 CD15 MPO CD11b CD67
granulocyt
CD13 CD15 MPO CD11b CD67 CD16
myeloide stamcel
HLA-DR CD34 (TdT) (CD13) (CD33) (CD7)
proërytroblast
H antigeen (Gp A)
erytroblast
H antigeen Gp A
erytrocyt
H antigeen Gp A
onrijpe megakaryoblast
HLA-DR CD34 CD33 (CD41/CD61) (CD42)
megakaryoblast
CD41/CD61 CD42 (CD9)
megakaryocyt
CD41/CD61 CD42 CD9
trombocyten
CD41/CD61 CD42 CD9

acterized by the expression of the CD10 antigen and the weak cytoplasmic expression of the μ Ig heavy chain (weak Cyμ) (7,14).

The T cell markers CD2 and CD7 are expressed at the cell surface in virtually all stages of T cell differentiation (15). Furthermore, cytoplasmic expression of the CD3 antigen (CyCD3) occurs during the early stages of T cell differentiation, while mature T cells express the CD3 antigen at their cell surface in close association with the TcR (TcR-CD3 complex) (15). The expression of the various other T cell markers (such as CD1, CD4, CD5 and CD8) is indicated in Figure 1 (15,16).

NK cells are negative for CyCD3 and the TcR-CD3 complex, but they are positive for the pan-T cell marker CD7 and generally also for the CD2 antigen. In addition, they express the CD16 antigen (low affinity receptor for IgG; FcγRIII) and the CD56 antigen (neural cell adhesion molecule; NCAM) (17,18). A subpopulation of the NK cells expresses the CD8 antigen and the CD57 antigen (human NK cell antigen; HNK-1) (17). It should be noted that expression of CD16, CD56 and CD57 molecules is not NK-cell-specific, but that these molecules can also be found on T lymphocytes, especially $CD8^+$ T lymphocytes (Figure 1).

Myeloid markers

Analogous to lymphoid differentiation, also during myeloid differentiation certain markers become expressed, while others disappear.

Almost all cells of the granulocytic and monocytic differentiation lineages express the pan-myeloid markers CD13 and CD33 as well as myeloperoxidase (MPO) (19,20). In addition, the monocytic cells express the CD14 antigen, while cells of the granulocytic lineage express the CD15 antigen. Most macrophages are positive for CD68 and RFD9, and mature granulocytes express CD16 (FcγRIII) and the CD67 antigen (5,6)

The H antigen (backbone of ABO blood group proteins) and glycophorin A (GpA) can be used as markers for cells of the erythroid lineage (21). The CD41/CD61 and CD42 molecules represent platelet glycoproteins, which can be used as markers for the megakaryocytic-platelet lineage (22-24).

Non-lineage-restricted markers

Some immunologic markers are expressed in several differentiation lineages, such as in case of HLA-DR and the complement receptor type 3 (CR3), i.e. the CD11b antigen. Furthermore, precursor cells of the lymphoid and myeloid lineages express the CD34 antigen (25). Several other non-lineage-restricted markers are given in Table 1 and Figures 1 and 2 (5,6).

Figure 2. Hypothetical scheme of myeloid differentiation. The expression of relevant immunologic markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and NHL and indicate where these malignancies can be located according to their maturation arrest: AML, acute myeloid leukemia (the codes for the various types of AML are derived from the French-American-Britisch classification (26,27)); AUL, acute undifferentiated leukemia; CML, chronic myeloid leukemia; NHL, non-Hodgkin lymphoma.

TABLE 1. Immunologic markers (CD codes and alternative names) which can be used for immunophenotyping of cells in the various stages of hematopoietic differentiation and their malignant counterparts.

Immature lymphoid marker	
TdT	terminal deoxynucleotidyl transferase (expression on nuclear membrane)
B cell markers	
CD10	common ALL antigen (gp100)
CD19	pan-B cell antigen (gp90)
CD20	B cell antigen (p35)
CD21	complement (C3d) receptor (CR2) (gp140)
CD22	pan-B cell antigen (gp135)
CD37	B cell antigen (gp40-52)
FMC7*	B cell antigen (gp105)
B-ly-7*	hairy cell marker (gp144)
weak Cyμ	weak cytoplasmic expression of Ig heavy chain μ in pre-B cells
SmIg	surface membrane expression of Ig (heavy and light chains)
CyIg	cytoplasmic expression of Ig (heavy and light chains)
T cell markers	
CD1	T6 antigen (gp43, gp45, gp49)
CD2	T11 antigen; leucocyte function antigen 2 (LFA-2) (gp50)
CD3	T3 antigen (consists of at least five chains) (gp16-25)
CyCD3	cytoplasmic expression of CD3 protein chains
CD4	T4 antigen (gp60)
CD5	T1 antigen (gp67)
CD7	Tp41 antigen (gp41)
CD8	T8 antigen (gp32)
TcR-CD3	expression of the T cell receptor-CD3 complex on the cell surface
NK cell markers	
CD56	neural cell adhesion molecule (NCAM) (gp220/135)
CD57	human NK cell antigen (HNK-1) (gp110)
Pan-myeloid markers	
CD13	pan-myeloid antigen (gp150)
CD33	pan-myeloid antigen (gp67)
MPO	myeloperoxidase (gp60/12)
Myeloid-granulocytic markers	
CD15	X hapten
CD67	granulocytic antigen (p100)
Monocyte-macrophage markers	
CD14	monocytic antigen (gp55)
CD68	macrophage antigen (gp110)
RFD9*	macrophage antigen (gp25)
Erythroid markers	
H antigen	backbone of ABO blood group proteins
GpA	glycophorin A (gp41)
Megakaryocyte-platelet markers	
CD41	GPIIb-GPIIIa complex (Glanzmann antigen) (gp145/115)
CD42	GPIX-GPIb complex (Bernard-Soulier antigen) (gp143/22/20)
CD61	GPIIIa (gp115); associated with GpIIb (see CD41)
Non-lineage-restricted markers	
CD9	p24 antigen
CD11b	complement (C3bi) receptor (CR3)
CD11c	adhesion molecule (p150,95)
CD16	low affinity Fc receptor for IgG (FcγRIII) (gp50-65)
CD25	interleukin-2 receptor (IL-2R) (gp55)
CD34	precursor antigen (gp115)
CD38	T10 antigen (gp45)
HLA-DR	MHC-class-II antigen (gp29/34)

* Non-clustered immunologic markers

IMMUNOLOGIC PHENOTYPES OF LEUKEMIAS AND NHL

Immunologic markers can not only be used for characterization of normal hematopoietic cells, but also for further characterization of their malignant counterparts, i.e. the various leukemias and NHL (1,2).

The maturation arrest of the acute leukemias is located in the immature differentiation stages, such as in case of the acute undifferentiated leukemias (AUL), acute lymphoblastic leukemias (ALL) and acute myeloid leukemias (AML) (26,27). In chronic leukemias the maturation-arrest is located in more mature differentiation stages (chronic lymphocytic leukemia (CLL), prolymphocytic leukemia (PLL), hairy cell leukemia (HCL) and chronic myeloid leukemia (CML)) (1,28). NHL generally have a mature immunologic phenotype, such as in case of centroblastic (CB) lymphomas, centroblastic-centrocytic (CB-CC) lymphomas, centrocytic (CC) lymphomas, immunoblastic lymphomas and the true histiocytic lymphomas (29,30). Also the cutaneous T cell leukemia lymphoma (CTLL; mycosis fungoides/Sézary syndrome) and the adult T cell leukemia lymphoma (ATLL) have a mature immunologic phenotype (1,29-31).
In Figures 1 and 2 we have tried to summarize where the various types of leukemias and NHL can be located according to their maturation-arrest. Their corresponding immunologic phenotypes can be read from these figures. It should be noticed that within a malignancy more than one differentiation stage can occur. This implies that not all cells of such a malignancy will express the same immunologic markers. It also indicates that malignant cells still have some capacity to mature and that a maturation arrest may involve more than one differentiation stage. Furthermore, loss of immunologic markers may occur in malignancies. These phenomena may explain discordances between immunologic and morphological classifications, which especially occur in AML and NHL (30,32).

DIAGNOSTIC APPLICATIONS OF IMMUNOLOGIC MARKER ANALYSIS

Immunophenotyping of leukemias and NHL in addition to routine cytomorphology is important for a detailed and reproducible classification of these malignancies (1,2). In 15 to 30% of cases this will lead to a more appropriate or more accurate diagnosis than by use of cytomorphology alone. It is also important to search for correlations between morphological features, immunologic phenotypes and chromosomal aberrations, since this may give more insight into oncogenesis (33). Furthermore, this combined approach may allow the recognition of subgroups of leukemias and NHL, which differ in clinical behavior and response to therapy (34). Therefore, it is worthwhile to classify leukemias and NHL on the basis of morphological, immunologic and cytogenetic (MIC) criteria in order to adapt treatment protocols in the future (35). Immunologic marker analysis can also be used for the detection of two hematopoietic malignancies in one patient or for the detailed characterization of subpopulations within one malignancy (36).

Finally, it should be remarked that immunologic markers can also be applicated for the detection of low numbers of malignant cells (37). During maintenance treatment and even after withdrawal of therapy it remains uncertain whether or not malignant cells are still present. The detection limit of conventional morphological techniques is not lower than 1 to 5% (1 to 5 malig-

nant cells between 100 normal cells), since small numbers of malignant cells are difficult to discriminate from normal cells. Also the application of immunologic marker analysis for the detection of low numbers of malignant cells is hampered by the presence of normal counterparts, which express the same markers. However, in specific cases it is possible to detect minimal disease, based on the assumption that the presence of positive cells outside their normal homing areas is indicative of malignancy (37). In this way TdT can be used as marker for the detection of ALL cells in cerebrospinal fluid. This allows the early detection (or exclusion) of central nervous system leukemia, which is a severe complication in ALL (38).

Another example is the detection of extremely low numbers of malignant cells in the bone marrow and blood of patients with a TdT positive T cell malignancy by use of a double labeling technique for a T cell marker and TdT. Due to its low detection limit (0.001-0.01%), this technique allows the recognition of a relapse 2 to 6 months earlier than by use of conventional cytomorphology (37).

CONCLUSION

Immunologic marker analysis of leukemias and NHL is an important supplementation in the diagnostics of these malignancies. It results in a precise and reproducible classification and also permits other applications, such as the detection of low numbers of malignant cells. These applications allow adjustment of diagnostic criteria as well as individualization of therapy, which is valuable for the prevention of undertreatment and overtreatment of patients.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof.Dr. R. Benner for his continuous support, Mr. C. van Dijk and Mr. V.T.M. Gribling (Audiovisueel Centrum of the Erasmus University) for graphic design, and Ms. J. de Goey-Van Dooren for typing this manuscript.

REFERENCES

1. Foon KA, Todd III RF. Immunological classification of leukemia and lymphoma. Blood 1986;68:1-31.
2. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.
3. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Human leucocyte differentiation antigens detected by monoclonal antibodies. Berlin: Springer-Verlag, 1984.
4. Reinherz EL, Haynes BF, Nadler LM, Bernstein ID, eds. Leucocyte typing II. Volume 1: Human T lymphocytes; Volume 2: Human B lymphocytes; Volume 3: Human myeloid and hematopoietic cells. Berlin: Springer-Verlag, 1986.
5. McMichael AJ, Beverley PCL, Gilks W, Horton M, Mason DY, Cobbold S, Gotch FM, Ling N, Milstein C, Waldmann H, Crumpton MJ, Hogg N, MacLennan ICM, Spiegelhalter D. Leucocyte typing III. White cell differentiation antigens. Oxford: Oxford University Press, 1987.
6. Knapp W, Dörken B, Rieber EP, Stein H, Gilks WR, Schmidt RE, Von dem Borne AEGKr. Leucocyte Typing IV. White cell differentiation antigens. Oxford: Oxford University Press, 1989.
7. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells

determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
8. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-5.
9. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.
10. Campana D, Janossy G, Bofill M, Trejdosiewicz LK, Ma D, Hoffbrand AV, Mason DY, Lebacq A-M, Forster HK. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985;134:1524-30.
11. Zola H. The surface antigens of human B lymphocytes. Immunol Today 1987;8:308-15.
12. Boué DR, LeBien TW. Expression and structure of CD22 in acute leukemia. Blood 1988;71:1480-6.
13. Visser L, Shaw A, Slupsky J, Vos H, Poppema S. Monoclonal antibodies reactive with hairy cell leukemia. Blood 1989;74:320-5.
14. Gathings WE, Lawton AR, Cooper MD. Immunofluorescent studies of the development of pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur J Immunol 1977;7:804-10.
15. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as a diagnostic marker for immature T-cell malignancies. Blood 1988;71:603-12.
16. Lanier LL, Allison JP, Phillips JH. Correlation of cell surface antigen expression on human thymocytes by multi-color flow cytometric analysis: implications for differentiation. J Immunol 1986;137:2501-7.
17. Lanier LL, Le AM, Phillips JH, Warner NL, Babcock GF. Subpopulations of human natural killer cells defined by expression of the Leu-7 (HNK-1) and Leu-11 (NK-15) antigens. J Immunol 1983;131:1789-96.
18. Lanier LL, Le AM, Civin CI, Loken MR, Phillips JH. The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes. J Immunol 1986;136:4480-6.
19. Griffin JD, Mayer RJ, Weinstein HJ, Rosenthal DS, Coral FS, Beveridge RP, Schlossman SF. Surface marker analysis of acute myeloblastic leukemia: identification of differentiation-associated phenotypes. Blood 1983;62:557-63.
20. Van der Schoot CE, Daams GM, Pinkster J, Vet R, Von dem Borne AEGKr. Monoclonal antibodies against myeloperoxidase are valuable immunological reagents for the diagnosis of acute myeloid leukaemia. Br J Haematol 1990;74:173-8.
21. Villeval JL, Cramer P, Lemoine F, Henri A, Bettaieb A, Bernaudin F, Beuzard Y, Berger R, Flandrin G, Breton-Gorius J, Vainchenker W. Phenotype of early erythroblastic leukemias. Blood 1986;68:1167-74.
22. Koike T, Aoki S, Maruyama S, Narita M, Ishizuka T, Imanaka H, Adachi T, Maeda H, Shibata A. Cell surface phenotyping of megakaryoblasts. Blood 1987;69:957-60.
23. San Miguel JF, Gonzalez M, Cañizo MC, Ojeda E, Orfao A, Caballero MD, Moro MJ, Fisac P, Lopez Borrasca A. Leukemias with megakaryoblastic involvement: clinical, hematologic, and immunologic characteristics. Blood 1988;72:402-7.
24. Breton-Gorius J, Villeval JL, Kieffer N, Mitjavila MT, Guichard J, Vainchenker W. Limits of phenotypic markers for the diagnosis of megakaryoblastic leukemia. Blood Cells 1989;15:259-77.
25. Tindle RW, Nichols RAB, Chan L, Campana D, Catovsky D, Birnie GD. A novel monoclonal antibody BI-3C5 recognises myeloblasts and non-B non-T lymphoblasts in acute leukaemias and CGL blast crises, and reacts with immature cells in normal bone marrow. Leuk Res 1985;9:1-9.
26. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnik HR, Sultan C. Proposals for the classification of the acute leukaemias. Br J Haematol 1976;33:451-8.
27. Bennett JM, Catovsky D, Daniel M-T, Flandrin G, Galton DAG, Gralnik HR, Sultan C. Criteria for the diagnosis of acute leukemia of megakaryocyte lineage (M7). Ann Intern Med 1985;103:460-2.
28. Caligaris-Cappio F, Janossy J. Surface markers in chronic lymphoid leukemias of B cell type. Semin Hematol 1985;22:1-12.
29. Tubbs RR, Fishleder A, Weiss RA, Savage RA, Sebek BA, Weick JK. Immunohistologic cellular phenotypes of lymphoproliferative disorders. Comprehensive evaluation of 564 cases including 257 non-Hodgkin's lymphomas classified by the international working formulation. Am J Pathol 1983;113:207-21.

30. Schuurman H-J, Van Baarlen J, Huppes W, Lam BW, Verdonck LF, Van Unnik JAM. Immunophenotyping of non-Hodgkin's lymphoma. Lack of correlation between immunophenotype and cell morphology. Am J Pathol 1987;129:140-51.
31. Weiss LM, Crabtree GS, Rouse RV, Warnke RA. Morphologic and immunologic characterization of 50 peripheral T-cell lymphomas. Am J Pathol 1985;118:316-24.
32. Drexler HG. Classification of acute myeloid leukemias. A comparison of FAB and immunophenotyping. Leukemia 1987;1:697-705.
33. Yunis JJ. The chromosomal basis of human neoplasia. Science 1983;221:227-36.
34. Sobol RE, Mick R, Royston I, Davey FR, Ellison RR, Newman R, Cuttner J, Griffin JD, Collins H, Nelson DA, Bloomfield CD. Clinical importance of myeloid antigen expression in adult acute lymphoblastic leukemia. N Engl J Med 1987;316: 1111-7.
35. First MIC cooperative study group. Morphologic, immunologic, and cytogenetic (MIC) working classification of acute lymphoblastic leukemias. Cancer Genet Cytogenet 1986;23: 189-97.
36. Hagemeijer A, Bolhuis RLH, Van Dongen JJM, Sizoo W. Simultaneous occurrence of acute monoblastic leukemia and an abnormal B-cell clone with both cell types characterized by specific cytogenetic and immunological markers. Scand J Haematol 1986;36:484-91.
37. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986;113-33.
38. Hooijkaas H, Adriaansen HJ, Van Dongen JJM. Detection of central nervous system involvement in patients with leukemia or non-Hodgkin's lymphoma by immunological marker analysis of cerebrospinal fluid cells. In: Bennett JM, Foon KA, eds. Immunologic approaches to the classification and management of lymphomas and leukemias. Dordrecht: Martinus Nijhoff Publishers, 1988;149-71.

CHAPTER 5.2

CYTOPLASMIC EXPRESSION OF THE CD3 ANTIGEN AS A DIAGNOSTIC MARKER FOR IMMATURE T-CELL MALIGNANCIES*

J.J.M. van Dongen[1], G.W. Krissansen[2], I.L.M. Wolvers-Tettero[1], W.M. Comans-Bitter[1,3], H.J. Adriaansen[1], H. Hooijkaas[1], E.R. van Wering[4], C. Terhorst[5]

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt, Rotterdam, The Netherlands;
2. The Imperial Cancer Research Fund, Lincoln's Inn Fields, London, UK;
3. Department of Pediatrics, Sophia Children's Hospital, Rotterdam, The Netherlands;
4. The Dutch Childhood Leukemia Study Group, The Hague, The Netherlands;
5. Laboratory of Molecular Immunology, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA.

SUMMARY

The expression of cytoplasmic CD3 (CyCD3) was analyzed in 45 leukemias, five thymus cell samples, five peripheral blood (PB) samples, and ten cell lines. All T cell acute lymphoblastic leukemias (T-ALL) that did not express surface membrane CD3 (SmCD3) appeared to express CyCD3. Furthermore, the majority of $SmCD3^+$ T-ALL also expressed CyCD3. Analogous results were obtained with thymus cell samples in that about 95% of the thymocytes expressed CyCD3 whereas 60 to 75% of the thymocytes also expressed SmCD3. In normal peripheral blood only prominent SmCD3 expression was found. These data indicate that immature T cells express CyCD3 only, that the combined expression of CyCD3 and SmCD3 is characteristic for intermediate differentiation stages and that mature T cells express prominent SmCD3. All (precursor) B cell leukemias, acute myeloid leukemias, and non-T cell lines tested did not express CyCD3. On the basis of these data, we conclude that CyCD3 expression is restricted to the T cell lineage and can be used as a diagnostic marker for immature $SmCD3^-$ T cell malignancies. Therefore, we evaluated which fixative is optimal for CyCD3 staining, and we determined by immunofluorescence staining and Western blotting which CD3 monoclonal antibody (McAb) can be used for the detection of CyCD3. In our opinion, acid ethanol was the best fixative for the cytocentrifuge preparations. Furthermore, we demonstrated that CyCD3 can be easily detected by use of McAb raised against denaturated CD3 chains such as those of the SP series (SP-6, SP-10, SP-64 and SP-78). In addition we tested 22 CD3 McAb of the Oxford CD3 panel that were raised against native SmCD3, and it appeared that only four (UCHT1, VIT-3b, G19-41 and SK7/Leu-4) of them were able to detect CyCD3. In Western blot analysis all four McAb recognized the CD3-ϵ chain only.

* *Published in: Blood 1988;71:603-12.*

INTRODUCTION

T cell differentiation and T cell ontogeny can be studied by analysis of thymus subpopulations (1-4) or, alternatively, by analysis of T cell leukemias because the latter can be regarded as the malignant counterparts of cells in the various T cell differentiation stages (5-8). Such studies revealed that the immunologic phenotype of T cells gradually changes during differentiation, finally resulting in mature T cells that express the CD3 antigen on their cell surface (1-10).

The human CD3 antigen consists of at least three protein chains (CD3-γ, CD3-δ and CD3-ϵ) (11-13), the encoding genes of which have been cloned recently (14-16). These CD3 chains are closely associated with the antigen-specific receptor of T cells, the so-called T cell receptor (TcR) (13,17,18). To date, three different TcR chains have been described: TcR-α, TcR-β, and TcR-γ (19). Like the immunoglobulin genes, the TcR genes rearrange during T cell differentiation to enable receptor diversity (19). The majority of the mature T cells express a TcR that is composed of disulfide-linked TcR-α and TcR-β chains (20,21). The TcR-γ chain is expressed by a subpopulation of thymocytes and a small population of blood lymphocytes that can display non-major histocompatibility complex-restricted cytotoxicity (22-26). In the TcR-CD3 complex on the cell membrane of mature T cells the CD3 antigen probably plays a role in transduction of signals from the TcR to the cytoplasm (21,27,28).

Link et al reported cytoplasmic expression of CD3 (CyCD3) by immature T cell leukemias as detected in frozen tissue sections and cytocentrifuge preparations, whereas no CD3 expression could be detected on the cell surface of these immature T cells by immunofluorescence (IF) staining in suspensions (29). At that time they could not explain their findings; however, recent studies indicate that CD3 expression on the cell surface is dependent on the presence of the TcR (30-32) and that CD3 mRNA is already present in immature T cells (33,34). Therefore, CyCD3 can be expected in immature T cells that do not yet express the CD3 antigen on the cell surface membrane (SmCD3). Such CyCD3 expression has recently been reported by Furley et al (33) and Campana et al (35).

In this study we evaluated the expression of CyCD3 during early T cell differentiation and proved its application as a diagnostic marker for immature T cell malignancies. Therefore, we determined by Western blotting and IF staining techniques which CD3 monoclonal antibodies (McAb) can be used for the detection of CyCD3.

MATERIALS AND METHODS

Cell samples

Leukemic cell samples.

Bone marrow (BM) and peripheral blood (PB) samples were obtained from 45 leukemia patients at diagnosis. These comprised 19 T cell acute lymphoblastic leukemias (T-ALL), one T cell chronic lymphocytic leukemia (T-CLL), 12 precursor B-ALL (null ALL, common ALL or pre-B-ALL), seven B-CLL, and six acute myeloid leukemias (AML).

Normal PB samples.

Five PB samples were obtained from five healthy adult volunteers.

Thymus samples.

Five thymus samples were obtained from five children (<2 y old) undergoing cardiac surgery. These samples were minced with scissors in RPMI 1640 medium containing 15% fetal calf serum (FCS) and were flushed through a nylon gauze filter with 100-μm openings (Stokvis and Smits Textielmij, Haarlem, The Netherlands).

All human cell samples were obtained with the approval of the Committee of Medical Ethics of the Academic Hospital, Rotterdam.

Cell lines.

For control experiments and for testing the various CD3 McAb, we used the T cell lines Molt4, H9, 8102, CEM, and HSB; the precursor B cell lines BV173 and SMS-SB; and the Burkitt's lymphoma-derived cell line ROS-1, ROS-15, and ROS-17. The cell lines were cultured in RPMI 1640 medium supplemented with 15% FCS and antibiotics.

Immunologic marker analysis

Mononuclear cells (MNC) from BM and PB were isolated by Ficoll-Paque (density, 1.077 g/ml; Pharmacia, Uppsala, Sweden) density centrifugation. All washings were performed in phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA), pH 7.8.

Routine immunologic marker analysis.

Routine immunologic marker analysis of the cell samples was performed by determining the nuclear membrane expression of terminal deoxynucleotidyl transferase (TdT), surface membrane expression of Ig (SmIg), and surface membrane expression of HLA-DR and various T cell markers, B cell markers, and myelomonocytic markers (8).

Positivity for TdT was tested on cytocentrifuge preparations (Cytofuge, Nordic Immunological Laboratories, Tilburg, The Netherlands) by using a rabbit anti-TdT antiserum and a fluorescein isothiocyanate (FITC)-conjugated, goat anti-rabbit Ig, second-step antiserum (Supertechs, Bethesda, MD) (36). Expression of SmIg was tested by incubation of cells in suspension with FITC or tetramethylrhodamine isothiocyanate (TRITC)-conjugated goat antihuman Ig antisera (anti-human κ light chain, anti-human λ light chain and anti-human μ heavy chain; Kallestad Laboratories, Austin, TX). Surface membrane expression of HLA-DR and the various T cell markers (CD1, CD2, CD3, CD4, CD5, CD7, CD8, and TcR-$\alpha\beta$), B cell markers (CD10, CD19, and CD20), and myelomonocytic markers (CD13, CD14, CD15, and CD33) was tested by incubation of cells in suspension with the following McAb: L243 (anti-HLA-DR, nonpolymorphic antigen), Leu-1 (CD5), Leu-2 (CD8), Leu-3 (CD4), Leu-4 (CD3), Leu-5 (CD2), (Becton Dickinson, Sunnyvale, CA), 66IIC7 (CD1; Dr. M. van de Rijn, Amsterdam), 3A1 (CD7; American Type Culture Collection, Rockville, MD), WT31 (recognizes a nonpolymorphic epitope on TcR-$\alpha\beta$; Dr. W. Tax, Nijmegen, The Netherlands), B1 (CD20), B4 (CD19), My4 (CD14), My7 (CD13), My9 (CD33) (Coulter Clone, Hialeah, FL), VIL-A1 (CD10), and VIM-D5 (CD15), (Dr. W. Knapp, Vienna) (6,8,37). When McAb were used, an FITC-conjugated, goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service (CLB), Amsterdam, The Netherlands) was used as a second-step reagent.

Detection of CyCD3 protein chains.

For the detection of CyCD3 protein chains, cytocentrifuge preparations were made, air-dried, fixed, and subsequently incubated with CD3-γ, CD3-δ, or CD3-ϵ antibodies. Several fixatives for fixation of the cytocentrifuge preparations were tested: methanol, ethanol, acetone, acid ethanol (ethanol with 5% (v/v) acetic acid), and formol acetone (acetone with 1% (v/v) formaldehyde).

The following anti-CD3 protein chain antibodies were used: a rat CD3-γ polyclonal antiserum (16), the CD3-δ McAb SP-64 and SP-78, and the CD3-ϵ McAb SP-6 and SP-10 (38). A FITC-conjugated, goat anti-rat Ig antiserum (Nordic) was used as a second-step reagent in the case of the rat antiserum and a FITC-conjugated, goat anti-mouse Ig antiserum in the case of the McAb.

Double IF staining for a surface membrane marker and an intracellular marker.

Double IF stainings were performed for SmIg and CyCD3 as well as for T cell membrane markers and TdT. For SmIg/CyCD3 double IF stainings, the cells in suspension were incubated with a TRITC-conjugated, anti-human Ig antiserum. Subsequently, cytocentrifuge preparation of these labeled cells were subjected to indirect IF staining with a CD3-δ or CD3-ϵ McAb and a FITC-conjugated, goat anti-mouse Ig antiserum as a second-step reagent. For T cell marker/TdT double IF stainings, the cells in suspension were incubated with an anti-T cell marker McAb and an TRITC-conjugated, goat anti-mouse Ig antiserum (CLB). Subsequently, cytocentrifuge preparations of these cells were subjected to indirect IF staining with the rabbit anti-TdT antiserum and the FITC-conjugated, goat anti-rabbit antiserum.

Microscopes.

Zeiss (Carl Zeiss, Oberkochen, FRG) and Leitz (Ernst Leitz Wetzlar, Wetzlar, FRG) microscopes were used for the evaluation of the IF staining. The microscopes were equipped with HBO mercury lamps (Osram, Berlin, FRG), phase contrast facilities, and filter combinations for the selective visualization of FITC and TRITC.

Western blotting

Immuno-affinity purified CD3 (0.1 nmol) from human tonsils (39) was loaded into a 10-cm well and electrophoresed in a 15% sodium dodecyl sulfate (SDS)-polyacrylamide gel (40). As was demonstrated previously, CD3-γ, CD3-δ, and CD3-ϵ chains can be separated from each other in SDS-polyacrylamide gel electrophoresis (41). The separated polypeptides were electrophoretically transferred at 4°C to a nitrocellulose filter (Schleicher and Schuell, Dassel, FRG) at 300 V for two hours in a Tris-glycine-methanol buffer (16). The filter was blocked overnight at 4°C with 2% (w/v) BSA, 5% (w/v) skim milk powder, 0.05% Tween 20, and 0.02% Na azide in PBS. Reactivity of the various CD3 McAb with the transferred CD3 polypeptides was studied by incubating strips of the nitrocellulose filter overnight at 4°C with the McAb at a dilution of 1:50 in PBS containing 2% (w/v) BSA and 0.02% Na azide. After washing three times in PBS and 0.05% Tween 20, the strips were incubated for 1.5 hours with a biotinylated rabbit anti-mouse Ig antiserum (Vector Laboratories, Burlingame, CA) and washed as described earlier. The strips were finally incubated for one hour with horseradish peroxidase-conjugated avidin (Vector). After washing, bound antibody was visualized with diaminobenzidine (0.5 mg/ml) and hydrogen peroxide (0.06%) in 10 mmol/l Tris-HCl buffer, pH 7.4.

CD3 McAb of the "Oxford CD3 panel"

The CD3 McAb, which were tested for reactivity with CD3 protein chains in Western blotting and for reactivity with CyCD3 in IF staining techniques, were derived from the CD3 panel of the Third International Workshop and Conference on Human Leucocyte Differentiation Antigens (Oxford, UK, September 1986). McAb 471 through 492 of the Oxford CD3 panel were tested: UCHT1 (471), X35-3 (472), XXIII.141 (473), XXIII.46 (474), XXIII.87 (475), 381 (476), VIT-3 (477), VIT-3b (478), BMA031 (479), BMA032 (480), BW239/347 (481), BW264/56 (482), 12F6 (483), G19-4.1 (484), T3(2ADA) (485), T3/2T8-2F4 (486), T3/RW2-8C8 (487), TE/RW2-4B6 (488), CLB-T3/3 (489), CRIS-7 (490), YTH12.5 (491) and SK7/Leu-4 (492).

RESULTS

Detection of CyCD3 protein chains

The McAb SP-6, SP-10, SP-64 and SP-78 (38) were used to determine which fixative should be used for the detection of CD3 protein chains in the cytoplasm of the T cell lines Molt4, H9, 8102, CEM, and HSB. Fixation of the cytocentrifuge preparations with acid ethanol (ethanol with

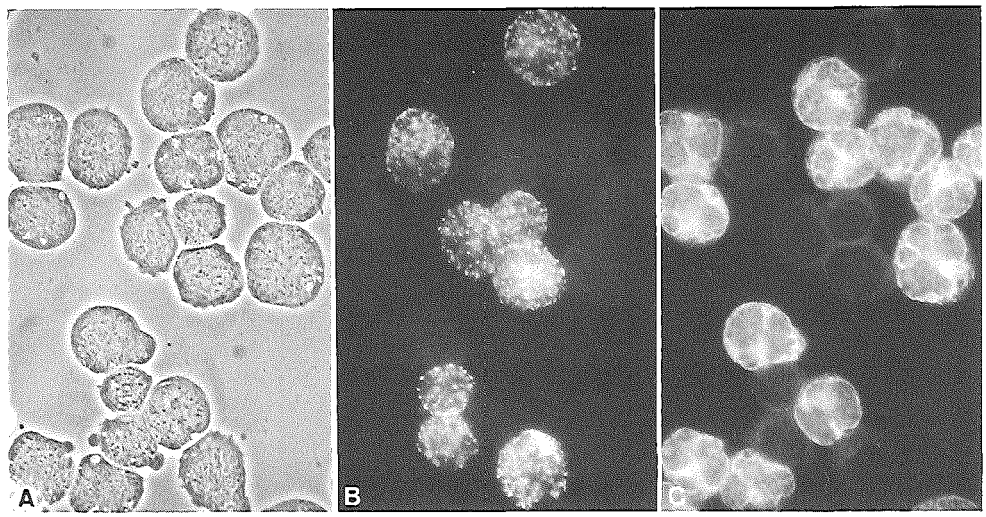


Figure 1. Double IF staining for SmIg and CyCD3 (SP-64) on a mixture of the Burkitt's lymphoma-derived cell line ROS-1 and the T cell line Molt4. **A**: Phase contrast morphology. **B**: $SmIg^+$ cells (TRITC labeled). **C**: $CyCD3^+$ cells (FITC labeled). The three micrographs represent the same field. All $SmIg^+$ cells are negative for CyCD3.

5% (v/v) acetic acid) for 15 minutes at –20°C appeared to result in the most prominent CyCD3staining. It should be remarked that only cytocentrifuge preparations with a good cytomorphology as determined by phase contrast microscopy resulted in optimal CyCD3 staining. Therefore, all cytocentrifuge preparations were checked for their quality before they were subjected to the CyCD3 staining procedure. Each of the four McAb of the SP series was able to detect CyCD3 chains in the five T cell lines tested. Also, surface membrane-like CD3 staining was observed in the cytocentrifuge preparations of the T cell lines H9 and CEM (see the next section).

To exclude nonspecific staining by the anti-CD3 chain McAb, an SmIg/CyCD3 double IF staining was performed on a 1:1 mixture of the T cell line Molt4 and the Burkitt's lymphoma-derived cell line ROS-1. In these experiments it appeared that all $SmIg^+$ cells were negative for CyCD3 and vice versa, thereby indicating that CyCD3 staining can be easily used for evaluation at the single-cell level (Figure 1).

CD3 staining patterns in cytocentrifuge preparations

The typical CyCD3 staining is a nondiffuse, irregular, filamentous staining that is most prominent at the position of nuclear clefts. This often results in a dotlike staining when a large nuclear cleft is present (see Figures 1, 2 and 3). Such information can be obtained only if the fluorescence microscope is equipped with optimally adjusted phase contrast facilities and if the cells are studied at various focus levels.

As was indicated earlier, the cells of the T cell lines H9 and CEM also expressed surface

TABLE 1. Immunologic marker analysis (including the detection of CyCD3 chains) of 19 T-ALL cell samples[a].

	T-ALL cell samples																		
Immunologic marker	1 (BM)	2 (BM)	3 (BM)	4 (PB)	5 (BM)	6 (BM)	7 (BM)	8 (BM)	9 (PB)	10 (BM)	11 (BM)	12 (PB)	13 (PB)	14 (PB)	15 (BM)	16 (BM)	17 (PB)	18 (BM)	19 (BM)
CyCD3-δ (SP-64)[b]	71	83	99	100	85	86	99	70	98	77	63	73	96[c]	99	60[c]	98[c]	0[d]	97	94[c]
CyCD3-ϵ (Sp-6)[b]	67	86	99	99	90	82	99	97	98	80	73	69	94[c]	98	59[c]	99[c]	0[d]	100	99[c]
TdT	89	26	96	87	58	87	81	74	99	95	58	80	95	87	64	79	69	96	99
HLA-DR	94	60	1	1	4	11	1	4	2	1	9	6	1	4	3	6	41	0	0
CD7 (3A1)	95	89 (86)	99	99	96 (95)	92	99	97	96	98	71 (99)	84	98	85	91	93	78	97	99
CD2 (Leu-5)	92	91 (68)	83	99	7 (0)	10	60	95	92	98	65 (96)	94	98	55	92	7	78	98	100
CD5 (Leu-1)	2	71 (61)	99	98	39 (48)	84	86	98	99	96	71 (89)	92	95	93	92	95	90	98	99
CD1 (66IIC7)	0	2 (0)	0	1	11 (18)	12	9	97	98	73	16 (22)	19	43	11	0	3	0	1	0
CD4 (Leu-3)	0	7 (0)	0	0	31 (59)	45	77	14	93	91	10 (3)	59	59	3	2	29	2	88	48
CD8 (Leu-2)	1	8 (1)	0	38	3 (0)	17	61	51	93	93	59 (99)	72	14	5	36	4	0	4	32
SmCD3 (Leu-4)[e]	4	3 (0)	1	0	2 (3)	1	2	11	2	12 (6)	24 (13)	68	83	57	67	67	95	82	87
TcR-αβ (WT31)	2	2 (0)	1	0	3 (0)	0	1	12	2	15 (0)	24 (4)	69	2	2	12(0)	7 (0)	1	23	68

a. The figures represent percent positivity per MNC. The figures in parentheses represent percent positivity per TdT^+ cells as detected by double IF staining; such T cell marker/TdT double IF stainings were primarily performed if the percentage of TdT^+ cells was lower than 60%.
b. Cytoplasmic staining as determined in cytocentrifuge preparation.
c. Both cytoplasmic and surface membrane-like staining were found.
d. Only surface membrane-like staining was found.
e. Surface membrane staining as determined by analysis of cells in suspension.

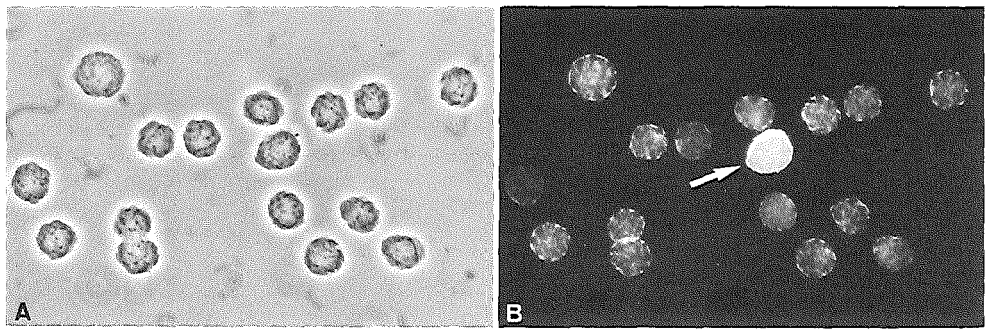


Figure 2. Staining for CD3 (SP-10) on a cytocentrifuge preparation of a thymus cell sample. **A**: Phase contrast morphology. **B**: CD3-positive cells. This figure illustrates different CD3 staining patterns: the majority of the thymocytes express both CyCD3 and SmCD3, whereas one cell (arrow) mainly expresses SmCD3.

membrane-like CD3 staining in addition to the typical CyCD3 staining. This was confirmed by CD3 staining of these cells in suspension. The SmCD3 staining pattern in cytocentrifuge preparations is dependent on the focus level and varies from ringlike (Figure 4), when the focus level is just above the object glass, to a more diffuse staining, when the focus level is somewhat higher (Figure 2). It should be remarked, however, that a weak CyCD3 staining may be undetectable in our assays when the SmCD3 staining is very bright.

Expression of CyCD3 by human thymocytes

Five thymocyte cell samples from children (<2 years old) were evaluated for the expression of CyCD3 by using the McAb of the SP series (38) and the rat CD3-γ antiserum. The majority (>95%) of the thymocytes were positive for the typical CyCD3 staining, and in addition, surface membrane-like staining was also seen on a major part of the thymocytes. The intensity of the CyCD3 and SmCD3 stainings varied from cell to cell. Three different types of CD3 staining patterns could be recognized in the thymus cell samples: about 20 to 30% of the thymocytes expressed only CyCD3, 60 to 75% expressed both CyCD3 and SmCD3, and about 5% of all thymocytes expressed prominent levels of SmCD3, whereas no typical CyCD3 staining was detectable in the latter cells (Figure 2). These findings indicate that the majority of $SmCD3^+$ thymocytes also contain variable amounts of CD3 protein chains in the cytoplasm.

Expression of CyCD3 by T-ALL cells

Nineteen T-ALL cell samples were analyzed for the expression of CyCD3-δ (SP-64 and SP-78) and CyCD3-ϵ (SP-6 and SP-10) as well as the expression of TdT and the surface membrane expression of the T cell markers CD1, CD2, CD3, CD4, CD5, CD7 and CD8; the B cell markers CD10, CD19, CD20; and the myeloid markers CD13, CD14, CD15 and CD33. The results of the immunologic marker analysis are summarized in Tables 1 and 2. All T-ALL

TABLE 2. Immunologic marker analysis (including the detection of CyCD3 chains) of 45 leukemias, ten cell lines, and five normal samples.

	Immunologic markers[a]														
Cell samples	TdT	HLA-DR	CD2 (Leu-5)	CyCD3-δ (SP-64)	CyCD3-ε (SP-6)	SmCD3 (Leu-4)	CD5 (Leu-1)	CD7 (3A1)	CD10 (VIL-A1)	CD13 (My7)	CD14 (My4)	CD15 (VIM-D5)	CD19 (B4)	CD20 (B1)	CD33 (My9)
$SmCD3^-$ T-ALL (n=11)	11/11	2/11	9/11	11/11[b]	11/11[b]	0/11	10/11	11/11	7/11	1/11[c]	0/10	0/11	1/11	0/10	1/11[d]
$SmCD3^+$ T-ALL (n = 8)	8/8	1/8	7/8	7/8[b]	7/8[b]	8/8	8/8	8/8	2/8	0/8	0/7	0/8	0/7	0/7	0/7
T-CLL (n = 1)	0/1	0/1	1/1	0/1[e]	0/1[e]	1/1	1/1	1/1	0/1	0/1	0/1	0/1	0/1	0/1	0/1
T cell lines (n = 5)	3/5	1/5	3/5	5/5[f]	5/5[f]	2/5	5/5	4/5	2/5	0/5	0/5	1/5	0/5	0/5	0/5
Precursor B-ALL (n = 12)	12/12	12/12	0/12	0/12	0/12	0/12	0/12	1/12	11/12	1/12	0/12	0/12	12/12	4/12	1/12
B-CLL (n = 7)	0/4	7/7	0/7	0/7	0/7	0/7	7/7	0/7	0/7	0/6	0/7	0/7	5/5	6/7	0/1
Precursor B cell lines (n = 5)	1/5	5/5	0/5	0/5	0/5	0/5	1/5	0/5	3/5	1/5	0/5	0/5	5/5	3/5	0/5
AML (n = 6)	1/5	6/6	1/6	0/6	0/6	0/6	0/6	3/6	0/6	3/6	2/6	1/6	1/6	0/6	6/6
Normal PB (n = 5)	<0.1	26±9	67±9	56±10[g]	60±7[g]	58±8	56±7	54±11	<0.1	17±9	15±7	1±2	7±2	6±2	NT[h]

a. The figures represent the number of positive leukemias or cell lines per total number of samples tested. A leukemia or cell line was considered to be positive if more than 25% of the MNC were positive. For the normal PB samples, percentages of positive cells ± SD are given.
b. In the $SmCD3^-$ T-ALL mainly cytoplasmic staining for CD3 chains was found, whereas in seven of the eight $SmCD3^+$ T-ALL samples both cytoplasmic and surface membrane-like staining was observed. One $SmCD3^+$ T-ALL sample did not express CyCD3 (see Table 1).
c. The $CD13^+$ T-ALL is the prothymocytic T-ALL of patient 1 (see Table 1 and Campana et al. (35) and Van Dongen et al. (42)).
d. The $CD33^+$ T-ALL is the immature thymocytic T-ALL of patient 2 (see Table 1).
e. No CyCD3 staining was detected; only SmCD3 staining was found.
f. In three of the five T cell lines, mainly cytoplasmic staining for CD3 was found (see Table 3).
g. These percentages represent surface membrane-like $CD3^+$ cells in the cytocentrifuge preparations; no typical cytoplasmic staining was detected.
h. NT, not tested.

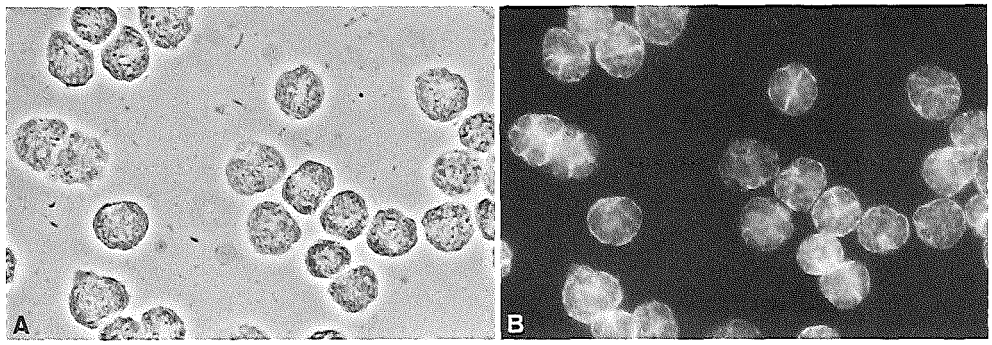


Figure 3. Expression of CyCD3 (SP-64) by an immature T-ALL (Table 1, patient 4). **A**: Phase contrast morphology. **B**: CD3-positive cells. All leukemic cells express CyCD3.

samples expressed TdT. If the percentage of TdT^+ cells was lower than 60%, double IF stainings for a T cell marker and TdT were performed to determine the immunologic phenotype of the TdT^+ cells (Table 1).

Eleven T-ALL samples did not express SmCD3 whereas eight T-ALL samples were positive for SmCD3. One $SmCD3^-$ T-ALL sample had the phenotype of the putative prothymocyte ($TdT^+/HLA\text{-}DR^+/CD7^+/CD2^+/CD5^-/CD1^-/CD3^-$), and six T-ALL samples appeared to be examples of immature thymocytic T-ALL ($TdT^+/CD7^+/CD2^+/CD5^+/CD1^-/CD3^-$) (34). All eleven $SmCD3^-$ T-ALL samples did express CyCD3, including the patient with prothymocytic T-ALL (Figure 3). Also seven out of eight T-ALL samples which expressed SmCD3, as determined by labeling of the cells in suspension, expressed CyCD3. These data probably indicate that all T-ALL samples, whether they are immature or mature, express either CyCD3, SmCD3, or both.

In addition, it should be noted that in five of the nine $SmCD3^+$ T-ALL samples almost no TcR-$\alpha\beta$ $(WT31)^+$ cells were detected, and if they were detected, it was demonstrated by double IF staining that the TdT^+ cells were negative for TcR-$\alpha\beta$ (WT31). In a sixth $SmCD3^+$ T-ALL a large discrepancy of about 60% was found between the percentages of SmCD3 positivity and TcR-$\alpha\beta$ (WT31) positivity. This may indicate that the T-ALL cells in these cell samples express the TcR-γ protein chain (22-26).

Analysis of 45 leukemias and ten cell lines for CyCD3 expression

The results of the immunologic marker analysis of the 45 leukemias and the ten cell lines are summarized in Table 2. All 19 T-ALL samples were positive for TdT and the CD7 antigen. In addition, they all expressed CyCD3 and/or SmCD3 as detected in cytocentrifuge preparations using CD3-δ and CD3-ϵ McAb (see the previous section and Tables 1 and 2). The T-CLL sample tested was negative for TdT but expressed the T cell markers CD2, CD3, CD5 and CD7 on the cell surface. The SmCD3 expression was confirmed by the surface membrane-like CD3 staining in cytocentrifuge preparations (Table 2). Three out of five T cell

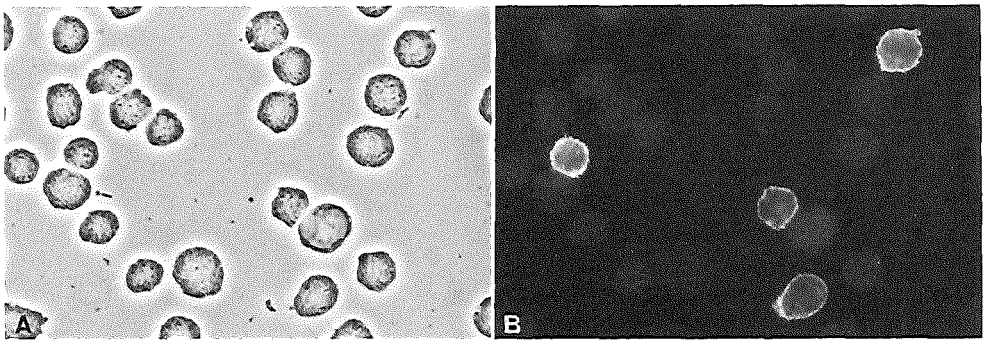


Figure 4. Staining for CD3 (SP-64) on a cytocentrifuge preparation of BM cells from a patient with a precursor B-ALL at diagnosis. **A**: Phase contrast morphology. **B**: CD3-positive cells. The leukemic blasts are negative for CyCD3, whereas a few cells express SmCD3. The latter cells represent the normal T lymphocytes. This figure illustrates the surface membrane-like CD3 staining in cytocentrifuge preparations.

lines were positive for TdT and mainly expressed CyCD3, whereas the other two T cell lines expressed both CyCD3 and SmCD3.

All 12 precursor B-ALL samples were positive for TdT, HLA-DR, and the B cell antigen CD19. Eleven out of 12 precursor B-ALL samples also expressed the CD10 antigen (common ALL antigen). These precursor B-ALL samples did not express CyCD3 or SmCD3. This is illustrated in Figure 4, which shows CD3 staining on a cytocentrifuge preparation of BM cells from a patient with a precursor B-ALL: the leukemic blasts were negative for CD3, whereas a few normal T lymphocytes (about 3% of the total MNC population) expressed SmCD3. The B-CLL samples tested were positive for HLA-DR, the B cell antigen CD19, and the T cell antigen CD5, and in addition they weakly expressed SmIg. The B-CLL samples did not express CyCD3 or SmCD3. The two precursor B cell lines and the three Burkitt's lymphoma-derived cell lines were positive for HLA-DR and the B cell antigen CD19, but they did not express CyCD3 or SmCD3. The six AML samples expressed HLA-DR and the myeloid antigen CD33. Three of these AML samples also expressed the myeloid antigen CD13, and three of them expressed the CD7 antigen. None of the AML samples were positive for CyCD3 or SmCD3.

These data indicate that the expression of CyCD3 and/or SmCD3 as detected in cytocentrifuge preparations by use of anti-CD3 chain McAb is restricted to cells of the T cell lineage.

Expression of CyCD3 and SmCD3 by MNC from PB

MNC from normal PB samples were analyzed for the expression of CyCD3 chains and SmCD3 as well as for several other T cell markers, B cell markers and myeloid markers (Table 2). The percentages of T lymphocytes varied from about 50 to 65%, whereas the percentage of B lymphocytes ($CD19^+$ or $CD20^+$) varied from about 5 to 10% and the monocytic cells ($CD14^+$) from about 10 to 20%.

The use of the anti-CD3 chain McAb in cytocentrifuge preparations of PB samples resulted

in surface membrane-like CD3 staining only. The typical CyCD3 staining could not be detected. The percentage of cells positive for SmCD3-δ and SmCD3-ϵ staining in the cytocentrifuge preparations were comparable with the percentages of $SmCD3^+$ cells as found by staining of the PB cells in suspension (Table 2).

Reactivity of the CD3 McAb of the Oxford panel in IF techniques and Western blotting

In addition to the rat CD3-γ antiserum (16), the CD3-δ McAb SP-64 and SP-78, and the CD3-ϵ McAb SP-6 and SP-10 (38), 22 CD3 McAb of the Oxford CD3 panel were evaluated for their capability to detect CyCD3 in cytocentrifuge preparations of the T cell lines Molt4, H9, and 8102. In addition, these McAb were tested for their reactivity with CD3 chains in Western blotting.

Detection of CyCD3 by the use of CD3 McAb of the Oxford panel.

The rat CD3-γ antiserum and the McAb of the SP series could detect CyCD3 chains in the three T cell lines (Table 3).

The McAb of the Oxford panel were first optimally titrated by testing their reactivity with SmCD3 on normal PB lymphocytes in suspension. Subsequently the McAb were tested for their capacity to detect CyCD3 in the T cell lines Molt4, H9, and 8102. Two different titers were used for this purpose, i.e. the titer that was optimal for the detection of SmCD3 as well as a fourfold-lower titer. It appeared that four CD3 McAb (UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4) were able to detect CyCD3 in all three T cell lines tested and that a fifth McAb, T3(2ADA), gave surface membrane-like CD3 staining in cytocentrifuge preparations of H9 and 8102. These five McAb did not show reactivity in cytocentrifuge preparations of B cell lines. The observation that Molt4 cells do not express SmCD3 may explain the absence of surface membrane-like staining in cytocentrifuge preparations stained with the McAb T3(2ADA); however, the surface membrane-like staining of the McAb T3(2ADA) in cytocentrifuge preparations of the $SmCD3^-$ cell line 8102 remains unexplained. The McAb VIT-3 caused a weak CyCD3 staining in cytocentrifuge preparations of Molt4 but did not react with H9 and 8102. Lowering of the titer of this McAb did not result in a better staining. The McAb T3/2T8-2F4 gave a nonspecific filamentous staining in all three T cell lines. The same nonspecific staining was also found in PB monocytes, as determined by phase contrast facilities, as well as in B cell lines. This filamentous staining was also described by Lobach et al (4) and resembles the filamentous staining of the CD3 McAb T10B9 as described by Campana et al (35).

These results indicate that the McAb UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4 are able to detect CyCD3 in all three T cell lines tested and that the McAb T3(2ADA) only gives surface membrane-like CD3 staining in cytocentrifuge preparations.

Reactivity of CD3 McAb of the Oxford panel with CD3 chains in Western blotting.

Antibodies were screened in Western blots against an immuno-affinity purified preparation of CD3 from human tonsils (39). An CD3-$\gamma\delta\epsilon$ polyclonal antiserum was used to determine the location of the CD3-γ, CD3-δ, and CD3-ϵ chains in the filter strips. As could be expected, the rat CD3-γ antiserum reacted with the CD3-γ band, whereas McAb SP-64 and SP-78 reacted with the CD3-δ band and McAb SP-6 and SP-10 with the CD3-ϵ band. All anti-CD3 McAb of the

TABLE 3. Reactivity of five anti-CD3 chain antibodies and 22 CD3 McAb from the Oxford CD3 panel as analyzed by the use of cytoplasmic IF staining and Western blotting.

	CD3 chain antibodies					McAb from the Oxford CD3 panel																					
	Rat CD3-γ antiserum	SP-64	SP-78	SP-6	SP-10	UCHT1	X35-3	XXIII.141	XXIII.46	XXIII.87	381	VIT-3	VIT-3b	BMA031	BMA032	BW239/347	BW264/56	12F6	G19-4.1	T3(2ADA)	T3/2T8-2F4	T3/RW2-8C8	TE/RW2-4B6	CLB-T3/3	CRIS-7	YTH12.5	SK7/Leu-4
Cytoplasmic IF staining[a] (cytocentrifuge preparations)																											
Molt4	+[b]	+	+	+	+	+	–	–	–	–	–	±	+	–	–	–	–	–	+	–	+[c]	–	–	–	–	–	+
H9	+	+	+	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	+	+[d]	+[c]	–	–	–	–	–	+
8102	+	+	+	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	+	+[d]	+[c]	–	–	–	–	–	+
Western blotting																											
CD3-γ band	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CD3-δ band	–	+	+	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
CD3-ε band	±[e]	–	–	+	+	+	–	–	–	–	–	–	+	–	–	–	–	–	+	+	–	–	–	–	–	–	+

a. A positive reaction implies mainly cytoplasmic staining unless otherwise indicated.
b. Abbreviations: +, positive reaction; ±, weak reaction; –, no reactivity.
c. Nonspecific staining of filaments.
d. Only surface membrane-like staining, no cytoplasmic staining.
e. The rat CD3-γ polyclonal antiserum showed a minor cross-reaction with the CD3-ε chain.

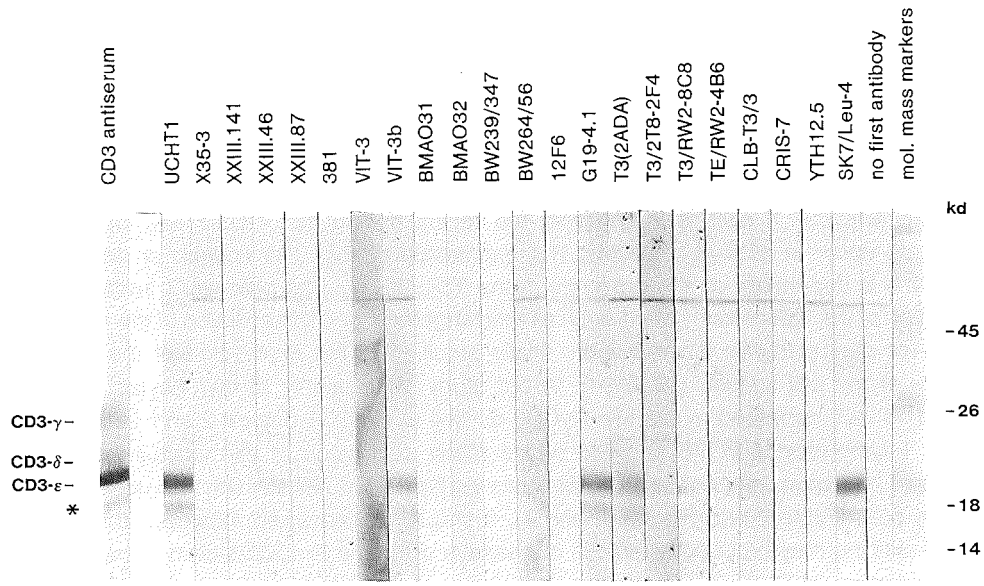


Figure 5. Western blot analysis of the CD3 McAb of the Oxford CD3 panel. The reactivity of a polyclonal CD3-γ δ ε antiserum, shown in the first filter strip, indicates the localization of the CD3-γ, CD3-δ, and CD3-ε bands. Only the McAb UCHT1, VIT-3b, G19-4.1, T3(2ADA) and SK7/Leu-4 show reactivity in the Western blot filter strips. They all recognize the CD3-ε band. The 18-kDa band migrating ahead of the CD3-ε band (*) is a specific degradation product of the CD3-ε polypeptide (39).

Oxford panel were tested at a titer of 1:50. Five McAb, (UCHT1, VIT-3b, G19-4.1, T3(2ADA) and SK7/Leu-4) showed reactivity with the CD3-ε band and an 18-kDa degradation product of this band (39), whereas all other McAb showed no reactivity (Figure 5).

Comparison of the data of the IF staining with the data of the Western blotting indicates that the five CD3 McAb of the Oxford panel that are able to give CyCD3 and/or surface membrane-like CD3 staining in cytocentrifuge preparations are the same McAb that react with the CD3-ε chains in Western blots (Table 3, Figure 5).

DISCUSSION

Expression of the CD3 antigen by a leukemia or non-Hodgkin's lymphoma proves the T cell origin of such a malignancy because the CD3 antigen is a T cell-specific marker (1,7,9,10). However, many T-ALL do not express the CD3 antigen on their surface membrane (9,10,33,34). Link et al demonstrated CD3 expression in the cytoplasm of some immature T cell malignancies that did not express SmCD3(29). Such CyCD3 expression in immature T cells has recently also been described by Furley et al (33) and Campana et al (35). Cytoplasmic expression of antigens preceding the surface membrane expression has also been described for other proteins such as the B cell antigen CD22 (43). Another well-known example is the weak

cytoplasmic expression of μ heavy chains in pre-B cells that precedes the SmIg expression on B lymphocytes (44,45).

Our study indicates that CyCD3 expression already occurs in immature stages of T cell differentiation because the majority of thymocytes and all immature T-ALL express CyCD3 (Table 1, Figures 2 and 3). Even the most immature T-ALL, i.e. the prothymocytic T-ALL, weakly expressed CyCD3 (Table 1). Expression of the CD3 antigen in the cytoplasm of immature $SmCD3^-$ T cells is in line with the recent finding that CD3-δ and CD3-ϵ gene transcripts occur in all T-ALL (33,34). The expression of the CD3 antigen on the surface membrane is dependent on the presence of the TcR (30-32). The processes involved in the production of the TcR protein chains such as rearrangement and transcription of the TcR genes take place during thymic differentiation, finally resulting in the expression of the TcR-CD3 complex at the cell surface (19,33,34). In our study, the majority of thymocytes and most $SmCD3^+$ T-ALL appeared to express CyCD3 as well as SmCD3 (Figure 2 and Table 1). The mature T lymphocytes in PB and BM and a minor population of thymocytes (about 5%) expressed prominent levels of SmCD3, whereas the typical CyCD3 staining was not detectable in these cells (Table 2 and Figures 2 and 4). It should be remarked, however, that a weak CyCD3 staining may be undetectable in our assays when the SmCD3 staining is very bright. Thus, immature $SmCD3^-$ T cells express CyCD3 only, the combined expression of CyCD3 and SmCD3 is characteristic for intermediate differentiation stages, and $SmCD3^+$ cells represent mature T cells. It might well be that the latter cells display a low CyCD3 expression because a continuous CD3 chain biosynthesis should compensate the SmCD3 turnover.

Whether the precursor T cells in BM, i.e. the prothymocytes, also express CyCD3 has to be further investigated. On the basis of double and triple immunologic stainings we have previously postulated that a minor population of the TdT^+ cells in BM represent prothymocytes that express the $TdT^+/HLA\text{-}DR^+/CD7^+/CD2^+/CD5^-/CD1^-/CD3^-$ phenotype (42,46). Recently, Campana et al performed double IF stainings for CyCD3 and TdT but could not detect double-positive cells in BM (35). Although we did not perform such double IF stainings on BM samples, we demonstrated that the prothymocytic T-ALL sample tested weakly expressed CyCD3 and in addition, we recently showed that such leukemic cells contain CD3-δ and CD3-ϵ gene transcripts (34).

The expression of the various T cell antigens, CyCD3 and the TcR-CD3 complex as well as the presence of CD3 transcripts during T cell differentiation are summarized in Figure 6.

To exclude the possibility that non-T cells expressed CyCD3, we analyzed 12 precursor B-ALL samples, seven B-CLL samples, five (precursor) B cell lines and six AML samples in addition to 19 T-ALL samples, one T-CLL sample and five T cell lines. Only the T cell leukemias and T cell lines appeared to express CyCD3 and/or SmCD3. All other non-T cell leukemia samples and non-T cell lines were negative for the CD3 antigen. Also the $CD7^+$ AML samples did not express CyCD3 (Table 2). These data and those of Campana et al (35) indicate that CyCD3 expression is restricted to the T cell lineage and can therefore be used as diagnostic marker for immature $SmCD3^-$ T cell malignancies.

For the detection of CyCD3, Link et al used the McAb Leu-4 on acetone-fixed tissue sections or cytocentrifuge preparations (29), Furley et al used McAb of the SP series but did not mention their fixation method (33), and Campana et al used the McAb UCHT1 and OKT3 on acetone-fixed cytocentrifuge preparations (35). Because the data discussed earlier indicated

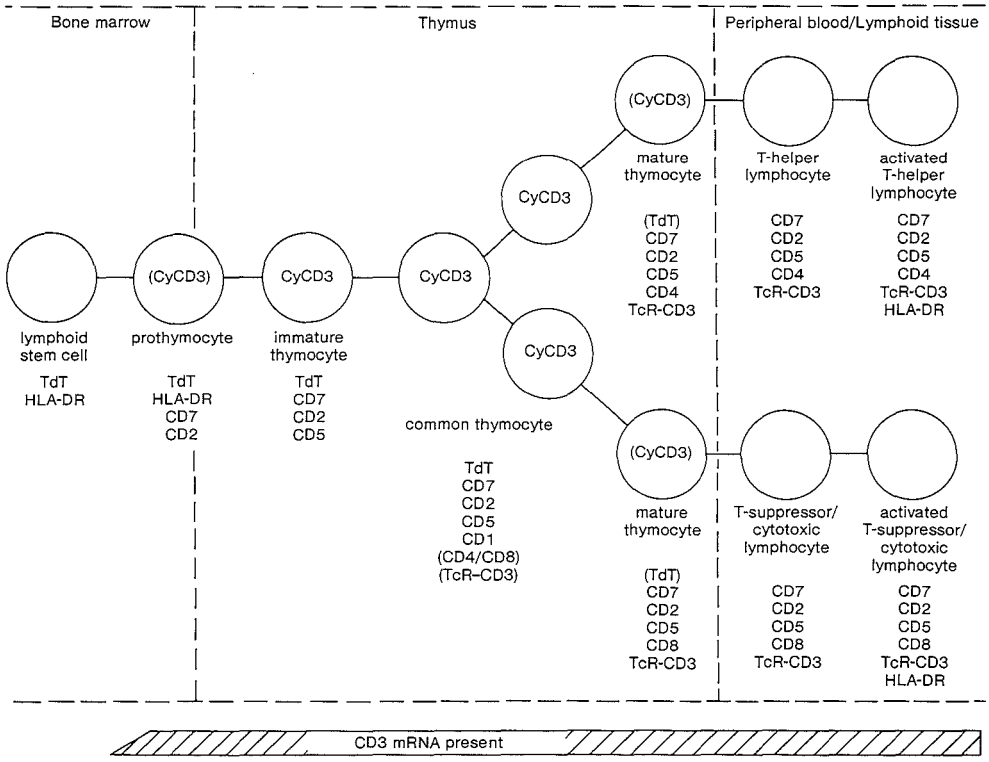


Figure 6. Hypothetical differentiation scheme of the T cell lineage that summarizes the expression of CyCD3, the TcR-CD3 complex, and other T cell markers in the various differentiation stages. Immunologic markers in parentheses are not always expressed. The dotted lines represent the tissue compartments, and the horizontal bar indicates the presence of CD3 mRNA (see Furley et al. (33) and Van Dongen et al. (34)).

that CyCD3 is an important diagnostic marker, we evaluated which fixation method is optimalfor the CyCD3 staining, and we determined which CD3 McAb can be used for the detection of CyCD3.

In our hands acid ethanol appeared to be the best fixative to fixate the cytocentrifuge preparations and to denature the CD3 chains. Our data and those of Furley et al (33) clearly demonstrate that CyCD3 expression can readily be detected by McAb of the SP series (38); However, the McAb SP-6 and SP-10 (CD3-ϵ) and the McAb SP-64 and SP-78 (CD3-δ) do not recognize SmCD3 on cells in suspension (38), and in addition, they are not widely available. Therefore, we wished to analyze the reactivity of CD3 McAb that are raised against native SmCD3. For this purpose we were allowed to use 22 CD3 McAb of the Oxford CD3 panel, kindly provided by professor A.M. McMichael. These McAb were tested for their capacity to detect CyCD3 in acid ethanol-fixed cytocentrifuge preparations of the T cell lines Molt4, H9 and 8102 as well as for their reactivity with CD3 chains in Western blotting.

Only five (UCHT1, VIT-3b, G19-4.1, T3(2ADA) and SK7/Leu-4) of 22 CD3 McAb of the Oxford CD3 panel could detect CD3 chains in cytocentrifuge preparations. Western blotting

showed that the same five McAb recognize the CD3-ϵ chain. All other CD3 McAb showed no reactivity with the CD3 chains in Western blotting. Four (UCHT1, VIT-3b, G19-4.1, and SK7/Leu-4) out of five mentioned McAb recognized both CyCD3 and SmCD3 in cytocentrifuge preparations, whereas use of the fifth one, T3(2ADA), resulted in surface membrane-like CD3 staining only (Table 3). In our opinion the McAb UCHT1 and SK7/Leu-4 gave the best CyCD3 staining.

The lack of reactivity of the majority of the CD3 McAb with denatured CD3 chains in cytocentrifuge preparations and Western blots may be due to the possibility that they recognize epitopes on the CD3 antigen that are composed of two or more chains or epitopes that change during denaturation. In view of these speculations, it is interesting to find that all five CD3 McAb of the Oxford panel that do detect CD3 in cytocentrifuge preparations recognize the CD3-ϵ chain. This may be connected with the fact that CD3-ϵ chains, in contrast to CD3-γ and CD3-δ chains, are not glycosylated (11,13). Another explanation may be that some epitopes on the CD3-ϵ chains are less prone to denaturation-induced changes.

Our results as a whole are consistent with CyCD3 being expressed by immature cells during T cell differentiation but not by precursor B cells and early myeloid cells. Thus CyCD3 can be used as a diagnostic marker for $SmCD3^-$ T cell malignancies. Evaluation of different CD3 McAb to be used for the detection of CyCD3 in cytocentrifuge preparations demonstrated that CyCD3 can be detected not only by the use of McAb raised against denatured CD3 chains such as those of the SP series but also by a minority of CD3 McAb that are raised against SmCD3, eg, UCHT1, VIT-3b, G19-4.1 and SK7/Leu-4.

Shortly before submission of this manuscript a report from Mirro et al. was published in which they described the detection of CyCD3 in five T-ALL cell samples by flowcytometry (47).

ACKNOWLEDGMENTS. We gratefully acknowledge Prof. Dr. R. Benner and Dr. M.J. Crumpton for their continuous support and advice; Prof. Dr. A.M. McMichael for providing us with the CD3 McAb of the Oxford CD3 panel; Dr. P.C.L. Beverly for providing us with the McAb UCHT1; Prof. Dr. E. Bos, MD, A.J. Meijer-Heijnsdijk, and colleagues for providing the thymus samples; Prof. Dr. J. Abels, MD, Drs. J. Burghouts, K. Hählen, A.C.J.M. Holdrinet, J.J. Michiels, J.C.M.B. Versteeg, J.C.M. Stigter, G.E. van Zanen, and their colleagues for providing the leukemia cell samples; B.E.M. van der Linden-Schrever for technical assistance; C.J. van Dijk and T.W. van Os for excellent photographic assistance; and J. de Goey-van Dooren and G. de Korte for help in preparing the manuscript.

This work was supported by the Netherlands Cancer Foundation (J.J.M. van Dongen and I.L.M.Wolvers-Tettero), the Sophia Foundation for Medical Research (H.J. Adriaansen) and National Institute of Health (C. Terhorst, grant AI 17651).

REFERENCES

1. Reinherz EL, Kung PC, Goldstein G, Levy RH, Schlossman SF. Discrete stages of human intrathymic differentiation: analysis of normal thymocytes and leukemic lymphoblasts of T-cell lineage. Proc Natl Acad Sci USA 1980;77:1588-92.
2. Tidman N, Janossy G, Bodger M, Granger S, Kung PC, Goldstein G. Delineation of human thymocyte differentiation pathways utilizing double-staining techniques with monoclonal antibodies. Clin Exp Immunol 1981;45:457-67.
3. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the

human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
4. Lobach DF, Hensley LL, Ho W, Haynes BF. Human T cell antigen expressing during the early stages of fetal thymic maturation. J Immunol 1985;135:1752-9.
5. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
6. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
7. Greaves MF. Differentiation-linked leukemogenesis in lymphocytes. Science 1986;234:697-704.
8. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology, Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.
9. Greaves MF, Rao J, Hariri G, Verbi W, Catovsky D, Kung P, Goldstein G. Phenotypic heterogeneity and cellular origins of T cell malignancies. Leuk Res 1981;5:281-99.
10. Chen P-M, Chiang H, Chou C-K, Hwang T-S, Chiang BN, Greaves MF. Immunological classification of T-cell acute lymphoblastic leukaemia using monoclonal antibodies. Leuk Res 1983;7:339-48.
11. Borst J, Alexander S, Elder J, Terhorst C. The T3 complex on human T lymphocytes involves four structurally distinct glycoproteins. J Biol Chem 1983;258:5135-41.
12. Oettgen HC, Kappler J, Tax WJM, Terhorst C. Characterization of the two heavy chains of the T3 complex on the surface of human T lymphocytes. J Biol Chem 1984;259:12039-48.
13. Borst J, Coligan JE, Oettgen H, Pessano S, Malin R, Terhorst C. The δ- and ϵ-chains of the human T3/T-cell receptor complex are distinct polypeptides. Nature 1984;312:455-8.
14. Van den Elsen P, Shepley B-A, Borst J, Coligan JE, Markham AF, Orkin S, Terhorst C. Isolation of cDNA clones encoding the 20K T3 glycoprotein of human T-cell receptor complex. Nature 1984;312:413-8.
15. Gold DP, Puck JM, Pettey CL, Cho M, Coligan J, Woody JN, Terhorst C. Isolation of cDNA clones encoding the 20K non-glycosylated polypeptide chain of the human T-cell receptor/T3 complex. Nature 1986;321:431-4.
16. Krissansen GW, Owen MJ, Verbi W, Crumpton MJ. Primary structure of the T3 γ subunit of the T3/T cell antigen receptor complex deduced from cDNA sequences: evolution of the T3 γ and δ subunits. EMBO J 1986;5:1799-1808.
17. Meuer SC, Fitzgerald KA, Hussey RE, Hodgdon JC, Schlossman SF, Reinherz EL. Clonotypic structures involved in antigen-specific human T cell function: relationship to the T3 molecular complex. J Exp Med 1983;157:705-19.
18. Brenner MB, Trowbridge IS, Strominger JL. Cross-linking of human T cell receptor proteins: association between the T cell idiotype β subunit and the T3 glycoprotein heavy subunit. Cell 1985;40:183-90.
19. Kronenberg M, Siu G, Hood LE, Shastri N. The molecular genetics of the T-cell antigen receptor and T-cell antigen recognition. Annu Rev Immunol 1986;4:529-91.
20. Meuer SC, Acuto O, Hercend T, Schlossman SF, Reinherz EL. The human T-cell receptor. Annu Rev Immunol 1984;2:23-50.
21. Weiss A, Imboden J, Hardy K, Manger B, Terhorst C, Stobo J. The role of the T3/antigen receptor complex in T-cell activation. Annu Rev Immunol 1986;4:593-619.
22. Brenner MB, McLean J, Dialynas DP, Strominger JL, Smith JA, Owen FL, Seidman JG, Ip S, Rosen F, Krangel MS. Identification of a putative second T-cell receptor. Nature 1986;322:145-9.
23. Bank I, DePinho RA, Brenner MB, Cassimeris J, Alt FW, Chess L. A functional T3 molecule associated with a novel heterodimer on the surface of immature human thymocytes. Nature 1986;322:179-81.
24. Lanier LL, Weiss A. Presence of Ti (WT31) negative T lymphocytes in normal blood and thymus. Nature 1986;324:268-70.
25. Borst J, Van de Griend RJ, Van Oostveen JW, Ang S-L, Melief CJ, Seidman JG, Bolhuis RLH. A T-cell receptor γ/CD3 complex found on cloned functional lymphocytes. Nature 1987;325:683-8.
26. Brenner MB, McLean J, Scheft H, Riberdy J, Ang S-L, Seidman JG, Devlin P, Krangel MS. Two forms of the T-cell receptor γ protein found on peripheral blood cytotoxic T lymphocytes. Nature 1987;325:689-94.
27. Samelson LE, Harford J, Schwartz RH, Klausner RD. A 20-kDa protein associated with the murine T-cell antigen receptor is phosphorylated in response to activation by antigen or concanavalin A. Proc Natl Acad Sci USA 1985;82:1969-73.

28. Cantrell DA, Davies AA, Crumpton MJ. Activators of protein kinase C down-regulate and phosphorylate the T3/T-cell antigen receptor complex of human T lymphocytes. Proc Natl Acad Sci USA 1985;82:8158-62.
29. Link MP, Stewart SJ, Warnke RA, Levy R. Discordance between surface and cytoplasmic expression of the Leu-4 (T3) antigen in thymocytes and in blast cells from childhood T lymphoblastic malignancies. J Clin Invest 1985;76:248-53.
30. Weiss A, Stobo JD. Requirement for the coexpression of T3 and the T cell antigen receptor on a malignant human T cell line. J Exp Med 1984;160:1284-99.
31. Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, Calman AF, Terhorst C, Stobo JD, Weiss A. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. Nature 1985;316:606-9.
32. Van Dongen JJM, Geurts van Kessel AHM, Wolvers-Tettero ILM, Versnel MA, Van Oudenaren A, Schoenmaker E, Hagemeijer A. Construction of human-mouse T cell hybrids that express human T cell-associated surface antigens and allow the chromosomal localization of these antigens. J Immunol 1986;137:1047-53.
33. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
34. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
35. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
36. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
37. Spits H, Borst J, Tax W, Capel PJA, Terhorst C, De Vries JE. Characteristics of a monoclonal antibody (WT-31) that recognizes a common epitope on the human T cell receptor for antigen. J Immunol 1985;135:1922-8.
38. Pessano S, Oettgen H, Bhan AK, Terhorst C. The T3/T cell receptor complex; antigenic distinction between the two 20-kd T3 (T3-δ and T3-ϵ) subunits. EMBO J 1985;4:337-44.
39. Krissansen GW, Verbi W, Totty NF, Crumpton MJ. Purification and identification by amino acid sequence analysis of the subunits of the CD3 antigen of human tonsil T-lymphocytes. Mol Immunol 1987;24:1069-79.
40. Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 1970;227:680-5.
41. Kanellopoulos JM, Wigglesworth NH, Owen MJ, Crumpton MJ. Biosynthesis and molecular nature of the T3 antigen of human thymocytes. EMBO J 1983;2:1807-14.
42. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
43. Campana D, Janossy G, Bofill M, Trejdosiewicz LK, Ma D, Hoffbrand AV, Mason DY, Lebacq A-M, Forster HK. Human B cell development. I. Phenotypic differences of B lymphocytes in the bone marrow and peripheral lymphoid tissue. J Immunol 1985;134:1524-30.
44. Gathings WE, Lawton AR, Cooper MD. Immunofluorescent studies of the development of the pre-B cells, B lymphocytes and immunoglobulin isotype diversity in humans. Eur J Immunol 1977;7:804-10.
45. Burrows P, LeJeune M, Kearney JF. Evidence that murine pre-B cells synthesise μ heavy chains but no light chains. Nature 1979;280:838-40.
46. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin De Jong J. Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.
47. Mirro J, Kitchingman G, Behm FG, Murphy SB, Goorha RM. T cell differentiation stages identified by molecular and immunologic analysis of the T cell receptor complex in childhood lymphoblastic leukemia. Blood 1987;69:908-12.

CHAPTER 5.3

DETECTION OF MINIMAL RESIDUAL ACUTE LYMPHOBLASTIC LEUKEMIA BY IMMUNOLOGIC MARKER ANALYSIS: POSSIBILITIES AND LIMITATIONS*

J.J.M. Van Dongen[1], H. Hooijkaas[1], H.J. Adriaansen[1], K. Hählen[2] and G.E. Van Zanen[2].

1. Department of Immunology, Erasmus University/University Hospital Dijkzigt Rotterdam;
2. Department of Pediatrics, Subdivision Hematology-Oncology,
Sophia Children's Hospital, Rotterdam, The Netherlands.

INTRODUCTION

The detection of small numbers of malignant cells is a major problem at diagnosis and during follow-up of malignancies (1). The possibilities and limitations of methods for the detection of minimal residual disease in general depend on their specificity (malignant cells have to be identified among many normal cells), sensitivity (detection limit of the method) and reproducibility. Although the method as such may have an excellent reproducibility, the cell sample may not always be representative for the whole cell population.

In leukemias and non-Hodgkin lymphomas (NHL) the detection limit for the identification of small numbers of malignant cells by means of morphological techniques is not lower than 1% (1 malignant cell among 100 normal cells). Therefore, many investigators have tried to use other techniques for the detection of small numbers of malignant cells, such as cytogenetics (2), cell culture systems (3), premature chromosome condensation (4) and recombinant DNA techniques (5-7). Most of these techniques, however, do not lower the 1% detection limit.

Both the normal hematopoietic differentiation stages and their malignant counterparts (i.e. leukemias and NHL) can be characterized by immunologic marker analysis (8-11). Some immunologic markers have been introduced as tumor-specific (12), but it turned out that these so-called "tumor markers" as well as most other immunologic markers represent differentiation antigens, which are also expressed by normal cells (8). Nevertheless, it is possible to use immunologic marker analysis by means of microscopy or flow cytometry for the detection of small numbers of malignant cells (1). Its application for the detection of minimal residual acute lymphoblastic leukemia (ALL) will be discussed.

---

* *Published in: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986:113-33. Updated for CD codes.*

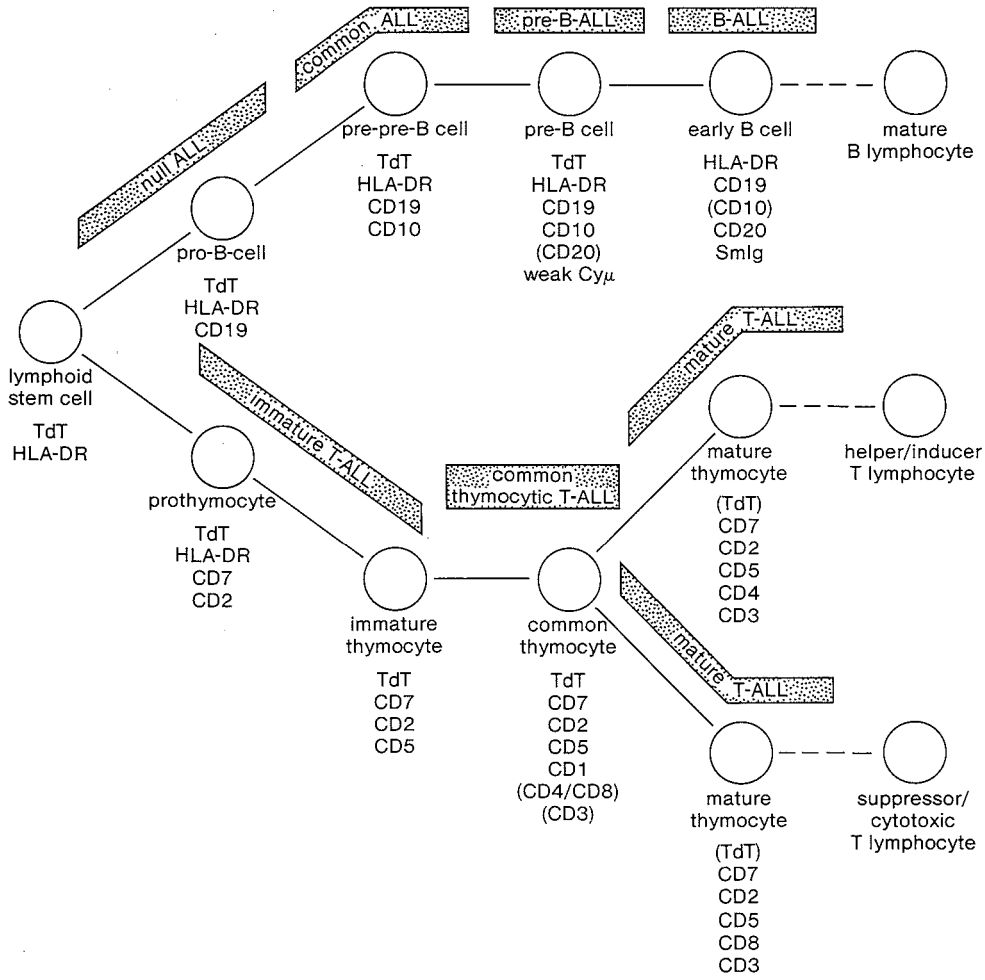


Figure 1. Hypothetical scheme of lymphoid differentiation. The markers expressed by cells in the various lymphoid differentiation stages are indicated. Markers in parenthesis are not always expressed. The bars represent malignant counterparts, i.e. the various (sub)types of ALL.

IMMUNOLOGIC PHENOTYPES OF ALL

ALL cells can be regarded as malignant counterparts of cells in immature lymphoid differentiation stages as indicated in Figure 1 (8,9). By use of immunologic marker analysis, at least five different immunologic phenotypes of ALL can be distinguished: null ALL, common ALL, pre-B-ALL, B-ALL and T-ALL (Table 1) (8,9,13). The T-ALL can be divided into several subtypes based on their positivity for different T cell markers (Figure 1) (14-18). Detailed information about the various immunologic markers for the characterization of ALL is summarized in Table 2 (19-32).

By use of recombinant DNA techniques it has been demonstrated that most null ALL, and

TABLE 1. Immunologic phenotypes of ALL.

	TdT	HLA-DR	CD19	CD10 (CALLA)	weak Cyμ	SmIg	T cell markers (CD2,CD7)
null ALL	+	+	(+)[a]	–	–	–	–
common ALL	+	+	+	+	–	–	–
pre-B-ALL	+	+	+	+	+	–	–
B-ALL	–	+	+	(+)[a]	–	+	–
T-ALL[b]	+	–[c]	–	–	–	–	+[b]

a. Not always expressed.
b. Several subtypes of T-ALL can be recognized, based on their positivity for the various T cell markers.
c. A few T-ALL are HLA-DR positive.

all common ALL and pre-B-ALL have rearranged immunoglobulin (Ig) heavy chain genes or rearranged Ig heavy and light chain genes (33-35), while most T-ALL have rearranged T cell receptor (TcR) β chain genes (7,35,36). However, some T-ALL appear to have rearranged Ig heavy chain genes (37). Similarly, some non-T-ALL have rearranged TcR-β chain genes (35). Despite lineage infidelities, the Ig gene and TcR gene rearrangement in general support the hypothesis that null ALL, common ALL and pre-B-ALL can be regarded as precursor B-ALL, as is indicated in Figure 1 (33-38).

IMMUNOLOGIC MARKER ANALYSIS FOR THE DETECTION OF MINIMAL RESIDUAL ALL

Although immunologic marker analysis can be used for a precise and reproducible immunologic classification of ALL (8,9), its application for the detection of small numbers of ALL cells is limited. This is mainly due to the presence of normal lymphoid cells which express the same immunologic markers (8,28,39,40). The frequencies of these normal cells influence the detectability of their malignant counterparts. Therefore, cells positive for certain markers or marker-combinations can only be regarded as malignant, if these positive cells are present in higher frequencies than normal or if they are detected outside their normal homing areas (Table 3).

For the detection of small numbers of ALL cells the following immunologic markers or marker combinations may be used: kappa-lambda ratio, terminal deoxynucleotidyl transferase (TdT), common ALL antigen (CD10), CD1 antigen and double immunofluorescence (IF) staining for TdT and a T cell marker.

Kappa-Lambda ratio

Since B cell malignancies are clonal expansions of one B cell they will express only one type of Ig light chain: kappa or lambda (23,24). Therefore, Ig light chain expression can be used as a marker of clonality and the kappa-lambda ratio can give an impression about the

TABLE 2. Immunologic markers for characterization of ALL.

Antibody/ technique	Cluster of differentiation[a]	Antigen recognized	Specificity	References
anti-TdT[b]	–	terminal deoxynucleotidyl transferase (TdT)	immature lymphoid cells	19
B4[c]	CD19	B cell antigen (p95)	all (precursor) B cells	20
VIL-A1[d]	CD10	common ALL antigen (CALLA) (gp100)	pre-pre-B cells, pre-B cells and subpopulation of B cells	21
BA-1[e]	CD24	B cell antigen (p45, 55,65)	most precursor B cells and all B cells	21
weak Cyμ[f]	–	weak expression of cytoplasmic μ heavy chain	pre-B cells	22
SmIg[f] (κ, λ, μ, δ, γ, α, ϵ)	–	surface membrane immunoglobulin	SmIg positive B cells	23,24
3A1[g] or WT1[h]	CD7	Tp41 antigen (p41)	all T cells	25,26
E (rosette)	"CD2"	sheep red blood cell (SRBC) receptor	all T cells	27
Leu-5[i] or T11[c]	CD2	T11 antigen; SRBC receptor (p50)	all T cells	28,29
Leu-1[i] or T1[c]	CD5	T1 antigen (p67)	thymocytes and mature T cells	28,29
Leu-6[i] or T6[c]	CD1	T6 antigen (common thymocyte antigen) (p45-12)	cortical thymocytes and Langerhans cells in the epidermis	28,29,30
Leu-3[i] or T4[c]	CD4	T4 antigen (p55)	T helper cells	28,29
Leu-2[i] or T8[c]	CD8	T8 antigen (p32-33)	T suppressor/cytotoxic cells	28,29
Leu-4[i] or T3[c]	CD3	T3 antigen (p19-29)	mature T cells	28,29
anti-HLA-DR[i]	–	HLA-DR, non polymorphic antigen (p19-34)	hematopoietic precursor cells and B cells	31,32

a. CD, cluster of differentiation as proposed by the "Leukocyte Typing Conference" (Boston, MA, 1984).
b. Bethesda Research Laboratories, Gaithersburg, MD.
c. Coulter Clone, Hialeah, FL.
d. New England Nuclear, Boston, MA.
e. Hybritech, San Diego, CA.
f. Kallestad Laboratories, Austin, TX.
g. American Type Culture Collection, Rockville, MD.
h. Dr. W. Tax, Nijmegen, The Netherlands.
i. Becton Dickinson, San Jose, CA.

TABLE 3. Detection of minimal residual ALL by immunologic marker analysis.

Limitations:
– some normal cells have the same immunologic phenotype as ALL cells
– the frequency of normal positive cells influences the detectability of their malignant counterparts
Possibilities:
– detection of positive cells in higher frequencies than in normal cell samples
– detection of positive cells outside their normal homing areas

number of malignant B cells (23,24,41,42). Due to the presence of normal polyclonal B cells, the detection limit of the kappa-lambda ratio analysis is not lower than 1 to 10%, even when large numbers of cells are evaluated by use of a sensitive flow-cytometric method (43,44). This kappa-lambda ratio analysis may be useful for detection of B-ALL, but not for other ALL, since only B-ALL expresses surface membrane Ig (SmIg).

Terminal deoxynucleotidyl transferase (TdT)

The enzyme TdT is present on the nuclear membrane of immature lymphoid cells of both B and T cell lineage as well as their malignant counterparts (precursor B-ALL and T-ALL) (8,45,46). TdT is a DNA polymerase lacking template requirements (45) and may play an important role in gene rearrangements (47), which are necessary for the transcription of Ig genes and TcR genes. The Ig gene rearrangements, occasionally found in acute myeloid leukemia (AML) (38), may therefore explain why some AML (5 to 10%) are TdT positive (48,49).

The majority of the TdT positive cells in bone marrow (BM) represents precursor B cells (8,39,45), while the TdT positive cortical thymocytes represent the precursors of the T lymphocytes (28,40,45,46). TdT positive cells are not only detectable in BM and thymus, but also in lower frequencies at other sites, such as peripheral blood (PB) and lymph nodes (LN) (45,50,51). For the detection of small numbers of TdT positive cells , the TdT biochemical assay is rather insensitive, and therefore the TdT immunofluorescence (TdT-IF) assay is preferred (45,52-55).

The frequencies of normal TdT positive cells in BM are age dependent and influenced by cytostatic therapy (45). Consequently, the percentages of TdT positive cells vary from about 1 to 6% and may even in regenerating BM increase up to 11% (Table 4). In PB the percentages of TdT positive cells are significantly lower and vary from about 0.01 to 0.06% and may be as high as 0.38% in some ALL patients during maintenance therapy (Table 4). TdT positive cells are also detectable in LN in frequencies of about 0.025% (Table 4). In cerebrospinal fluid (CSF) of fifty patients who did not suffer from ALL, we could not detect TdT positive cells among 100 to 1000 cells per sample evaluated. In some CSF samples from ALL patients in complete remission according to morphological criteria while receiving maintenance therapy, we detected TdT positive cells in low frequencies (<1%) (56,57). Most probably, such TdT positive cells represent ALL cells, but induction and maintenance therapy may eliminate these ALL cells or suppress their further proliferation.

TABLE 4. Frequencies of normal TdT positive cells in BM, PB and LN.

	Percentages of TdT positive cells		
	BM	PB	NL
Children (2 to 16y)	3.4 ± 2.5[a] (0.1-8.0)[b] (n=17)[c]	0.036 ± 0.033 (0.005-0.11) (n=14)	NT[d]
Adults	1.1 ± 0.5 (0.5-2.0) (n=8)	0.018 ± 0.014 (0-0.045) (n=8)	0.025 ± 0.029 (0-0.09) (n=15)
Children under ALL maintenance therapy	2.0 ± 1.6 (0.1-7.3) (n=174)	0.063 ± 0.074 (0-038) (n=221)	NT
Children, 6 wk after withdrawal of maintenance therapy	6.0 ± 2.8 (0.5-10.2) (n=8)	0.027 ± 0.007 (0.018-0.035) (n=3)	NT

a. Mean ± SD.
b. Range of percentage of positive cells.
c. Number of cell samples tested.
d. NT, not tested.

The normal "background" of TdT positive cells in BM and to a lesser extent in PB and LN, limits the value of the TdT-IF assay for detection of minimal residual ALL cells (Tables 4 and 5). However, Buchanan and colleagues (50,51) were able to detect elevated percentages of TdT positive cells in PB of ALL patients 3 to 5 wk prior to relapse. Their data indicate that the TdT-IF assay, performed on sequential PB samples, may be useful in detecting imminent relapse, if the PB is sampled at intervals of 2 to 4 wk. Barr et al. could not confirm these results (58). However, their PB sampling was performed less frequent and they evaluated lower numbers of cells. In addition they were hindered by the presence of non-specific fluorescent cells. This might have been due to their TdT-IF assay, since they did not fixate the cells and used anti-TdT antisera, which to our experience give high background staining (58,59).

Based on our data the detection limit of the TdT-IF assay for the identification of ALL cells in PB is about 0.4% (Table 5). Whether this rather high detection limit is valuable for prediction of all TdT positive ALL relapses, has to be investigated. Additional analysis of the TdT positive cells by double IF staining such as CD10/TdT or T cell marker/TdT may be a more appropriate approach.

Several studies indicate that the TdT-IF assay is valuable for improving the accuracy of the diagnosis of extramedullary ALL, e.g. by analysis of CSF and testis biopsies (56,57,60-62). This assay can be used for all TdT positive ALL, i.e. precursor B-ALL (null ALL, common ALL and pre-B-ALL) and T-ALL.

TABLE 5. Frequencies of normal positive cells as determined by immunologic marker analysis and their relevance for the detection limits of the various types of ALL cells.

Immunologic marker analysis	Normal ratio or frequency of positive cells	Detection limit of minimal residual ALL	ALL type
Kappa-lambda ratio	0.8-2.0	1-10%	B-ALL
TdT staining	BM: 1-6% (sometimes up to 11%) PB: 0.01-0.06% (sometimes up to 0.38%) LN: 0.025% (sometimes up to 0.09%) CSF: no TdT positive detectable in non-ALL patients[a]	BM: >11% PB: 0.4% LN: 0.1% CSF: ?[a]	TdT positive ALL (null ALL, common ALL pre-B-ALL and T-ALL)
CD10 staining	BM: 0.5-5%[b] (in regenerating BM up to 50%)[c] PB: adults: <0.002%[b] children under maintenance therapy: 0.002-0.014%[b] (sometimes up to 0.025%)	BM: ?[c] PB: 0.03%[b]	CD10 positive ALL (common ALL, pre-B-ALL and some B-ALL)
T cell marker/TdT double IF staining:			
– CD7/TdT and CD2/TdT staining	BM: 0.002-0.05% (sometimes up to 0.3%) PB: 0.001-0.015% (sometimes up to 0.02%)	BM: 0.04% PB: 0.03%	prothymocytic ALL ($CD2^+/CD7^+$/HLA-DR^+/TdT^+) and other TdT positive T cell malignancies
– CD5/TdT and CD1/TdT staining	BM: 0% PB: 0%	BM: at least 0.01% PB: at least 0.01%	most T-ALL and TdT positive T-NHL

a. Most probably all TdT positive cells in CSF (without PB or BM contamination) represent ALL cells.
b. Percentages CD10 positive lymphoid precursor cells, as determined by CD10/TdT double IF stainings (see text).
c. Due to high percentages CD10 positive cells in regenerating BM, no general detection limit for CD10 single IF staining can be determined. Only CD10/TdT double IF staining will be informative.

Common ALL antigen (CD10 antigen)

Initially, the CD10 antigen was regarded as leukemia specific (12). However, several investigators demonstrated CD10 positive cells in normal BM and fetal hematopoietic tissues (8,39,63,64). Especially in regenerating BM high percentages of CD10 positive cells are present (63). In the mononuclear cell (MNC) fraction of PB only extremely low numbers of CD10 positive cells are found (63,65). The CD10 antigen is also weakly expressed by granulocytes

(66) and by a subpopulation of thymocytes (67) and their malignant counterparts (15,18,68). According to our experience, especially CD1 positive T-ALL cells express the CD10 antigen.

The use of the CD10 antigen as an immunologic marker for monitoring BM of ALL patients for early detection of relapse is hampered by the presence of normal CD10 positive cells in frequencies of 0.5 to 5% and sometimes even up to 50% in regenerating BM (63). Especially due to the high frequency of normal CD10 positive cells in regenerating BM of ALL patients after cessation of maintenance therapy, it was concluded that monitoring of CD10 positive cells in BM has no predictive value with respect to relapse (63). However, according to our experience, it is possible to discriminate between a relapse and regeneration, since in regenerating BM the percentages of CD10 positive cells markedly exceed the percentages of TdT positive cells, while at relapse of a CD10 positive ALL the percentages of CD10 positive cells are generally lower than, or equal to the percentages of TdT positive cells.

CD10 positive lymphoid cells in PB are rare. It is difficult to detect these cells, since granulocytes are weakly positive for the CD10 antigen and CD10 monoclonal antibodies may nonspecifically bind to normal PB cells (63,66). Therefore, Ryan and colleagues tried to determine the frequency of normal CD10 positive lymphoid cells in PB by use of multiple flowcytometric parameters to exclude a variety of mature blood cells such as monocytes and granulocytes (65). By means of mixture experiments they demonstrated that it is possible to detect one CD10 positive lymphoid cell between 100,000 PB-MNC. The normal range of CD10 positive cells in adult PB appeared to be less than 16 per million PB-MNC. Based on these data it was concluded that the low normal background of CD10 positive cells may permit early detection of ALL relapse by analysis of the PB cells (65).

We have determined the background of normal CD10 positive cells in 90 PB samples from pediatric patients with ALL under maintenance therapy by double IF staining for the CD10 antigen and TdT using fluorescence microscopy. The frequency of normal $CD10^+/TdT^+$ cells in the PB of children under maintenance therapy appeared to be 0.002 to 0.014% (sometimes up to 0.025%). Based on the described data of Ryan and colleagues and our own data, we conclude that the detection of CD10 positive lymphoid cells by flowcytometry or CD10/TdT double IF staining in the PB of ALL patients in frequencies higher than 0.03%, may be indicative of an imminent relapse (Table 5). Further investigations have to substantiate the value of this PB monitoring.

The CD10 antigen can also be used as immunologic marker for the detection of malignant cells in CSF. This application proved to be helpful for the diagnosis of meningeal involvement in ALL patients (69).

Nevertheless it should be noted that the use of the CD10 antigen as immunologic marker for the detection of low numbers malignant cells in PB or CSF will only be valuable in CD10 positive ALL if no phenotypic shift occurs. Loss or diminution of CD10 expression as well as acquisition of CD10 expression have been described (70).

CD1 antigen

The CD1 antigen is present on about 70% of the thymocytes but is also expressed by Langerhans cells in the epidermis and by cells in tonsils and lymph nodes (30,71,72). CD1 positive cells in PB and regenerating BM have also been reported (73,74), but the precise

frequency and nature of these cells is still not known. By double IF staining it was demonstrated that the extrathymic CD1 positive cells are TdT negative (46,75,76).

It has been suggested that the CD1 antigen may be useful for the detection of minimal residual disease in patients with a CD1 positive T-ALL. However, it has to be remarked that the CD1 positive ALL represent only one third of T-ALL (17) and that phenotypic shift in CD1 expression at relapse has been described (77).

Double IF staining for a T cell marker and TdT is a better approach for detection of low numbers of T-ALL cells (75,78).

Double IF staining for a T cell marker and TdT

Several studies indicate that cells positive for both a T cell marker and TdT are normally present in the thymus only and not in normal or regenerating BM and PB (8,39,46,52,76,78). The T cell marker/TdT phenotype is also expressed by T-ALL (8,9,14-18) and some T cell NHL (T-NHL), especially lymphoblastic lymphomas (79-81). Therefore, it was concluded that the detection of T cell marker$^+$/TdT$^+$ cells in extrathymic locations is indicative of malignant T cells (8,52,78). Our experience with this assay will be discussed in the next section.

T CELL MARKER/TdT DOUBLE IF STAINING FOR DETECTION OF MALIGNANT T CELLS

To evaluate the possibilities and limitations of the double IF staining for the detection of malignant T cells we first determined the detection limit of this technique and investigated whether normal T cell marker$^+$/TdT$^+$ cells indeed do not occur in BM and PB (75,82). We use this double IF staining for detection of minimal disease at diagnosis and during follow-up.

Detection limit of the double IF staining

To determine the detection limit of the T cell marker/TdT double IF staining, we have performed several dilution experiments. Thymocytes or CD1$^+$/TdT$^+$ T-NHL cells were diluted with a suspension of normal PB-MNC. A double IF staining for the CD1 antigen and TdT was performed. It proved to be possible to detect one CD1$^+$/TdT$^+$ cell among 10,000 or even 100,000 normal cells. The detection limit of the double IF staining method is therefore at least 0.01% (75).

Normal T cell marker$^+$/TdT$^+$ in BM and PB

To investigate whether normal T cell marker$^+$/TdT$^+$ cells occur in BM and PB, over fifty BM and PB samples from children as well as adults were analyzed by use of the double IF staining. CD7, CD2, CD5 and CD1 antigens (Figure 1 and Table 2) were used as T cell markers in the double IF staining. CD5$^+$/TdT$^+$ or CD1$^+$/TdT$^+$ cells were not detected in any of the BM and PB samples analyzed. In contrast with data of other investigators (46,47), CD7$^+$/TdT$^+$ and

$CD2^+/TdT^+$ cells were detected in normal childhood BM and in BM from children under maintenance therapy in low frequencies: 0.02 to 0.05% (sometimes up to 0.3%) (Table 5). In adult BM and regenerating childhood BM, $CD7^+/TdT^+$ and $CD2^+/TdT^+$ cells were not always detectable, and if detected, the frequency was about five to ten-fold lower. Occasionally, $CD7^+/TdT^+$ cells could be detected in PB in a frequency of 0.001 to 0.015% (sometimes up to 0.02%) (Table 5). By use of triple immunologic staining for HLA-DR, the CD7 antigen and TdT (83), all $CD7^+/TdT^+$ cells appeared to be positive for HLA-DR. Since most hematopoietic precursor cells are positive for HLA-DR (32) and since the CD7 and CD2 antigens are pan-T cell markers (25,26,28,29), the extrathymic $CD7^+/HLA\text{-}DR^+/TdT^+$ cells and $CD2^+/TdT^+$ cells probably represent prothymocytes (82).

The presence of a background of normal $CD7^+/TdT^+$ and $CD2^+/TdT^+$ cells increases the detection limit of the T cell marker/TdT double IF staining when the CD7 and CD2 antigens are used as T cell markers. Consequently, the detection limit for prothymocytic ALL ($CD7^+/CD2^+/HLA\text{-}DR^+/TdT^+$) is about 0.4% in BM and 0.03% in PB (Table 5). Most T-ALL express additional T cell markers such as the CD5, CD1, CD4, CD8 and CD3 antigens (9,14-18), which are not expressed by normal extrathymic TdT positive cells (46,75,76,82). Therefore, the T cell marker/TdT double IF staining is very useful for the detection of minimal residual disease in patients with TdT positive T cell malignancies.

Use of the T cell marker/TdT double IF staining for monitoring BM and PB of patients with TdT positive T cell malignancies

The T cell marker/TdT double IF staining is routinely performed in our laboratory for the detection of low numbers of malignant cells in patients with T-ALL or TdT positive T-NHL at diagnosis as well as during follow-up (Figure 2). To prevent misdetection or underdetection of malignant cells due to phenotypic shift, we use two or three different T cell markers in parallel for the T cell marker/TdT double IF staining. The choice of these T cell markers is partly dependent on the immunologic phenotype of the malignancy at diagnosis.

Until now (October 1985) we have monitored the BM and PB of twelve children with a TdT positive T cell malignancy: nine T-ALL patients and three T-NHL patients. Three T-ALL patients had one or two relapses. By use of the double IF staining, it was possible to detect these relapses 2 to 3 months earlier than by use of conventional cytomorphological techniques only. In the BM and PB of the other nine children no malignant cells could be detected during follow-up; these patients are still in remission according to morphological as well as clinical criteria.

The results of CD5/TdT double IF staining on the BM and PB cells of a T-ALL patient during follow-up are summarized in Figure 3. After 6 weeks of induction therapy this patient was in remission according to morphological criteria (less than 5% blast cells in BM and no blast cells in PB). However, at that time still very low percentages of $CD5^+/TdT^+$ cells were detected in BM and PB. These percentages increased during follow-up, resulting in a morphological relapse about 3 months later. The patient received reinduction therapy and obtained remission according to morphological as well as immunologic criteria. After 10 weeks $CD5^+/TdT^+$ cells were again detected in PB. A second morphological relapse occurred two months later. This relapse proved to be therapy resistant.

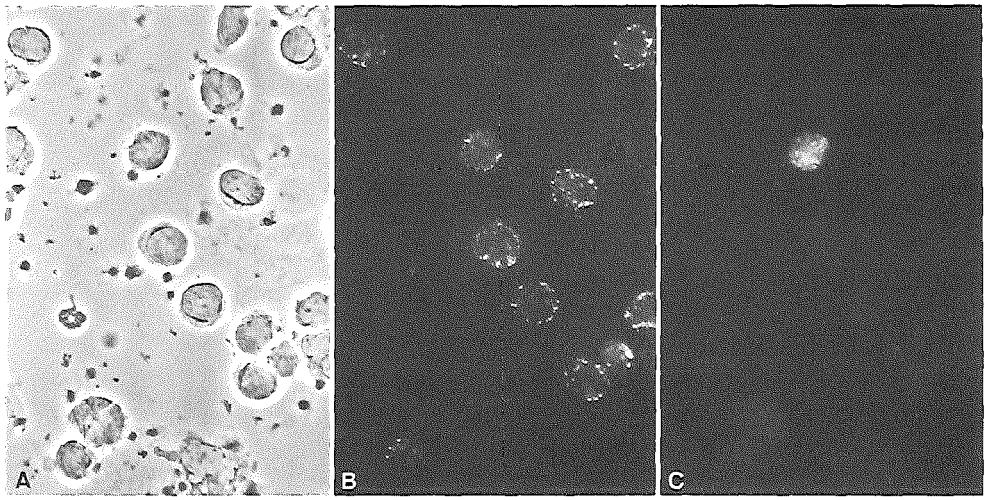


Figure 2. CD4/TdT double staining on PB cells from a T-ALL patient. **A**: phase contrast morphology; **B**: CD4 antigen positive cells (rhodamine labeled); **C**: TdT positive cell (fluorescein labeled). The TdT positive cell is also positive for the CD4 antigen and represents a T-ALL cell, while the other CD4 positive cells represent normal helper/inducer T lymphocytes.

Based on our follow-up data the following conclusions can be drawn:

- Very low numbers of malignant cells (about 0.01%) give rise to a morphological relapse 2 to 3 months later. This is in line with the results of animal experiments, in which injection of extremely low numbers of leukemic cells appeared to be able to cause a lethal leukemia (84,85).
- The increase of percentages $CD5^+/TdT^+$ cells in PB mimics the increase of $CD5^+/TdT^+$ cells in BM (Figure 3c), suggesting that the positive findings in PB reflect the process in BM. Therefore frequent PB sampling may be an useful alternative for frequent BM sampling during follow-up.
- The time interval between the detection of the immunologic relapse and the morphological relapse is shorter in the second relapse as compared to the first relapse. These data suggest that the proliferation capacity and/or therapy resistance of the T-ALL cells during the second relapse is higher than during the first relapse. This indicates that detection of low numbers of malignant cells can give information about both growth kinetics and the degree of therapy resistance. Furthermore, the double IF staining allows analysis of tumor regression during therapy, especially in the induction phase. Such analyses may turn out to be useful, if the kinetics of tumor regression has prognostic value.

Applications of the T cell marker/TdT double IF staining

Our results demonstrate that the T cell marker/TdT double IF staining can be used during follow-up of patients with a TdT positive T cell malignancy, to determine whether remission is

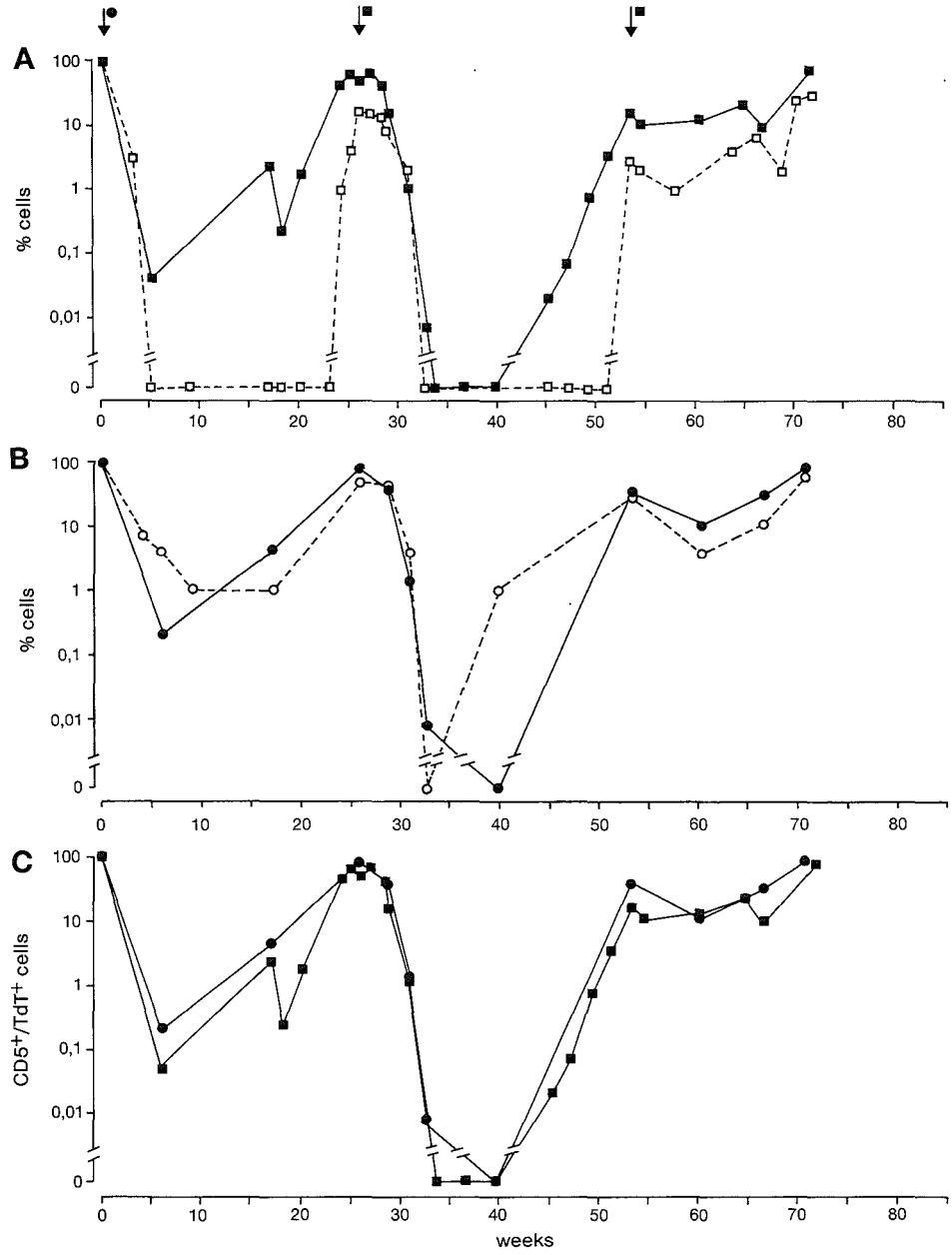


Figure 3. Follow-up of a T-ALL patient by morphological techniques and CD5/TdT double IF staining. *Solid squares*: $CD5^+/TdT^+$ cells in PB; *Open squares*: blast cells in PB; *Solid circles*: $CD5^+/TdT^+$ cells in BM; *Open circles*: blast cells in BM; $\downarrow^{\bullet}$: induction therapy; $\downarrow^{\blacksquare}$: reinduction therapy. The three figures summarize data of **A**: $CD5^+/TdT^+$ cells and blast cells in PB; **B**: $CD5^+/TdT^+$ cells and blast cells in BM; **C**: comparison between the percentages $CD5^+/TdT^+$ cells in BM and PB.

TABLE 6. Applications of the T cell marker/TdT double IF staining.

1. During follow-up:
– confirmation of remission after induction therapy
– early detection of relapse
– exclusion of relapse
2. Staging of TdT positive T-NHL at diagnosis
3. Autologous BM transplantation:
– detection of residual malignant cells in the BM graft

obtained during induction therapy, how fast this remission is obtained, and to detect a relapse at an early stage. In addition, it is possible to exclude an imminent relapse, when many suspect blast cells are detected by use of cytomorphological techniques, such as in regenerating BM (Table 6).

The T cell marker/TdT double IF staining can also be used for staging of TdT positive T-NHL at diagnosis (75,86). We analyzed BM and PB samples of three T-NHL patients at diagnosis, who had a stage I or stage II NHL according to morphological criteria. By use of double IF staining, malignant T cells were detected in low frequencies in all BM and PB samples. Therefore stage IV classification was more appropriate (Table 6). Our data underline the tendency of these T-NHL to disseminate and therefore explain why local treatment is insufficient in the majority of patients with mediastinal lymphoblastic lymphoma (87).

Finally, the T cell marker/TdT double IF staining can be used for analysis of the BM graft from patients with a TdT positive T cell malignancy, who undergo autologous BM transplantation. Since our data indicate that very low numbers of malignant cells can give rise to a relapse, it is important to exclude that malignant T cells are still present in the autologous BM graft (Table 6).

CONCLUSION

Detection of low numbers of ALL cells is possible by use of immunologic marker analysis. However, the detection limit is often not lower than the detection limit of cytomorphological techniques (1 to 10%) (Table 5). This is mainly due to a background of normal cells which express the same markers or marker-combination as the ALL cells. This normal background is age dependent and is influenced by cytostatic therapy.

Especially the detection limit of precursor B-ALL and B-ALL cells is limited by the normal background, which is, however, low in PB. As a consequence, TdT and the CD10 antigen may be useful for monitoring PB in precursor B-ALL patients (Table 5). In addition, TdT can be used as a marker for the detection of meningeal involvement in patients with a TdT positive ALL.

The best results are obtained in case of TdT positive T cell malignancies by use of the T cell marker/TdT double IF staining. The detection limit of this technique is very low: at least 0.01% for most T-ALL and TdT positive T-NHL (Table 5). Such low detection limits allow

adjustment of remission and staging criteria as well as individualization of therapy, which is valuable for the prevention of undertreatment as well as overtreatment of patients.

ACKNOWLEDGMENTS. We gratefully acknowledge Prof.Dr. R. Benner for his continuous support, Mr. M.W.M. van den Beemd and Mrs. K. Benne, W.M. Comans-Bitter, E.G.M. Cristen, I. Dekker, A.A. van der Linde-Preesman, A.F. Wierenga-Wolf, I.L.M. Wolvers-Tettero and J.H.F.M. Wijdenes-de Bresser for their technical assistance, Mr. C.J. van Dijk and Mr. T.M. van Os for their excellent photographic assistance and Mrs. C.J.M. Meijerink-Clerkx for typing the manuscript.

This work was supported by the Netherlands Cancer Foundation.

REFERENCES

1. Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Boston Martinus Nijhoff Publishers, 1984.
2. Sonta SI, Sandberg AA. Chromosomes and causation of human cancer and leukemia: XXVIII. Value of detailed chromosome studies on large numbers of cells in CML. Am J Hematol 1977;3:121-6.
3. Benjamin D, Magrath IT, Douglass EC, Corash LM. Derivation of lymphoma cell lines from microscopically normal bone marrow in patients with undifferentiated lymphomas: evidence of occult bone marrow involvement. Blood 1983;61:1017-9.
4. Hittelman WN, Menegaz SD, McCredie KB, Keating MJ. Premature chromosome condensation studies in human leukemia: 5 Prediction of early relapse. Blood 1984;64:1067-73.
5. Arnold A, Cossman J, Bakhshi A, Jaffe ES, Waldmann TA, Korsmeyer SJ. Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J Med 1983;309:1593-9.
6. Cleary ML, Chao J, Warnke R, Sklar J. Immunoglobulin gene rearrangement as a diagnostic criterion of B-cell lymphoma. Proc Natl Acad Sci USA 1984;81:593-7.
7. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T. Somatic rearrangement of T-cell antigen receptor gene in human T-cell malignancies. Proc Natl Acad Sci USA 1985;82:1224-7.
8. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
9. Foon KA, Schroff RW, Gale RP. Surface markers on leukemia and lymphoma cells: recent advances. Blood 1982;60:1-19.
10. Knapp W, ed. Leukemia markers. London: Academic Press, 1981.
11. Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Berlin: Springer Verlag, 1984.
12. Ritz J, Pesando JM, Notis-McConarty J, Lazarus H, Schlossman SF. A monoclonal antibody to human acute lymphoblastic leukaemia antigen. Nature 1980;283:583-5.
13. Van der Reijden HJ, Van Wering ER, Van de Rijn JM, Melief CJM, Van 't Veer MB, Behrendt H, Von dem Borne AEGK. Immunological typing of acute lymphoblastic leukemia. Scand J Haematol 1983;30:356-66.
14. Greaves MF, Rao J, Hariri G, Verbi W, Catovsky D, Kung P, Goldstein G. Phenotypic heterogeneity and cellular origins of T cell malignancies. Leuk Res 1981;5:281-99.
15. Bernard A, Boumsell L, Reinherz EL, Nadler LM, Ritz J, Coppin H, Richard Y, Valensi F, Dausset J, Flandrin G, Lemerle J, Schlossman SF. Cell surface characterization of malignant T cells from lymphoblastic lymphoma using monoclonal antibodies: Evidence for phenotypic differences between malignant T cells from patients with acute lymphoblastic leukemia and lymphoblastic lymphoma. Blood 1981;57:1105-10.
16. Catovsky D, Linch DC, Berverley PCL. T cell disorders in haematological diseases. In: Janossy G, ed. Clinics in Haematology. London: WB Saunders Company Ltd, 1982;11:661-95.
17. Roper M, Crist WM, Metzgar R, Ragab AH, Smith S, Starling K, Pullen J, Leventhal B, Bartolucci AA, Cooper MD. Monoclonal antibody characterization of surface antigens in childhood T-cell lymphoid malignancies.

Blood 1983;61:830-7.
18. Chen PM, Chiang H, Chou CK, Hwang TS, Chiang BN, Greaves MF. Immunological classification of T-cell acute lymphoblastic leukaemia using monoclonal antibodies. Leuk Res 1983;7:339-48.
19. Goldschneider I, Gregoire KE, Barton RW, Bollum FJ. Demonstration of terminal deoxynucleotidyl transferase in thymocytes by immunofluorescence. Proc Natl Acad Sci USA 1977;74:734-8.
20. Nadler LM, Anderson KC, Marti G, Bates M, Park E, Daley JF, Schlossman SF. B4, a human B lymphocyte-associated antigen expressed on normal, mitogen-activated, and malignant B lymphocytes. J Immunol 1983;131:244-50.
21. McKenzie IFC, Zola H. Monoclonal antibodies to B cells. Immunol Today 1983;4:10-5.
22. Vogler LB, Crist WM, Bockman DE, Pearl ER, Lawton AR, Cooper MD. Pre-B-cell leukemia: a new phenotype of childhood lymphoblastic leukemia. N Engl J Med 1978;298:872-8.
23. Hijmans W, Schuit HRE. Immunofluorescence studies on immunoglobulins in the lymphoid cells of human peripheral blood. Clin Exp Immunol 1972;11:483-94.
24. Teodorescu M, Mayer EP. Surface immunoglobulin in immunoproliferative diseases. Ann Clin Lab Sci 1978;8:353-65.
25. Haynes BF, Mann DL, Hemler ME, Schroer JA, Shelhamer JH, Eisenbarth GS, Strominger JL, Thomas CA, Mostowski HS, Fauci AS. Characterization of a monoclonal antibody that defines an immunoregulatory T cell subset for immunoglobulin synthesis in humans. Proc Natl Acad Sci USA 1980;77:2914-8.
26. Vodinelich L, Tax W, Bai Y, Pegram S, Capel P, Greaves MF. A monoclonal antibody (WT1) for detecting leukemias of T-cell precursors (T-ALL). Blood 1983;62:1108-13.
27. Moore AL, Zusman J. Conditions enhancing the percentage and size of E rosettes formed by T lymphocytes. J Immunol Methods 1978;23:275-84.
28. Janossy G, Prentice HG. T cell subpopulations, monoclonal antibodies and their therapeutic applications. In: Janossy G, ed. Clinics in Haematology. London: WB Saunders Company Ltd, 1982;11:631-60.
29. Hansen JA, Martin PJ, Beatty PG, Clark EA, Ledbetter JA. Human T lymphocyte cell surface molecules defined by the workshop monoclonal antibodies ('T cell protocol'). In: Bernard A, Boumsell L, Dausset J, Milstein C, Schlossman SF, eds. Leucocyte typing. Berlin: Springer Verlag, 1984:195-212.
30. Fithian E, Kung P, Goldstein G, Rubenfeld M, Fenoglio C, Edelson R. Reactivity of Langerhans cells with hybridoma antibody. Proc Natl Acad Sci USA 1981;78:2541-4.
31. Lampson LA, Levy R. Two populations of Ia-like molecules on a human B cell line. J Immunol 1980;125:293-9.
32. Falkenburg JHF, Jansen J, Van der Vaart-Duinkerken N, Veenhof WFJ, Blotkamp J, Goselink HM, Parlevliet J, Van Rood JJ. Polymorphic and monomorphic HLA-DR determinants on human hematopoietic progenitor cells. Blood 1984;63:1125-32.
33. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
34. Ford AM, Molgaard HV, Greaves MF, Gould HJ. Immunoglobulin gene organisation and expression in haemopoietic stem cell leukaemia. EMBO J 1983;2:997-1001.
35. Tawa A, Hozumi N, Minden M, Mak TW, Gelfand EW. Rearrangement of the T-cell receptor β-chain gene in non-T-cell, non-B-cell acute lymphoblastic leukemia of childhood. N Engl J Med 1985;313:1033-7.
36. Royer HD, Acuto O, Fabbi M, Tizard R, Ramachandran K, Smart JE, Reinherz EL. Genes encoding the Ti β subunit of the antigen/MHC receptor undergo rearrangement during intrathymic ontogeny prior to surface T3-Ti expression. Cell 1984;39:261-6.
37. Kitchingman GR, Rovigatti U, Mauer AM, Melvin S, Murphy SB, Stass S. Rearrangement of immunoglobulin heavy chain genes in T cell acute lymphoblastic leukemia. Blood 1985;65:725-9.
38. Rovigatti U, Mirro J, Kitchingman G, Dahl G, Ochs J, Murphy S, Stass S. Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia. Blood 1984;63:1023-7.
39. Janossy G, Bollum FJ, Bradstock KF, McMichael A, Rapson N, Greaves MF. Terminal transferase-positive human bone marrow cells exhibit the antigenic phenotype of common acute lymphoblastic leukemia. J Immunol 1979;123:1525-9.
40. Tidman N, Janossy G, Bodger M, Granger S, Kung PC, Goldstein G. Delineation of human thymocyte

differentiation pathways utilizing double-staining techniques with monoclonal antibodies. Clin Exp Immunol 1981;45:457-67.
41. Ligler FS, Vitetta Es, Smith RG, Himes JB, Uhr JW, Frenkel EP, Kettman JR. An immunologic approach for the detection of tumor cells in the peripheral blood of patients with malignant lymphoma; implications for the diagnosis of minimal disease. J Immunol 1979;123:1123-6.
42. Samoszuk MK, Krailo M, Yan QH, Lukes RJ, Parker JW. Limitations of numerical ratios for defining monoclonality of immunoglobulin light chains in B-cell lymphomas. Diagnostic Immunol 1985;3:133-8.
43. Ault KA. Detection of small numbers of monoclonal B lymphocytes in the blood of patients with lymphoma. N Engl J Med 1979;300:1401-5.
44. Smith BR, Weinberg DS, Robert NJ, Towle M, Luther E, Pinkus GS, Ault KA. Circulating monoclonal B lymphocytes in non-Hodgkin's lymphoma. N Engl J Med 1984;311:1476-81.
45. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
46. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
47. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-5.
48. Janossy G, Hoffbrand AV, Greaves MF, Ganeshaguru K, Pain C, Bradstock KF, Prentice HG, Kay HEM, Lister TA. Terminal transferase enzyme assay and immunological membrane markers in the diagnosis of leukaemia: a multiparameter analysis of 300 cases. Br J Haematol 1980;44:221-34.
49. Modak MJ, Mertelsmann R, Koziner B, Pahwa R, Moore MAS, Clarkson BD, Good RA. A micromethod for determination of terminal deoxynucleotidyl transferase (TdT) in the diagnostic evaluation of acute leukemias. J Cancer Res Clin Oncol 1980;98:91-104.
50. Froehlich TW, Buchanan GR, Cornet JAM, Sartain PA, Smith RG. Terminal deoxynucleotidyl transferase-containing cells in peripheral blood: implications for the surveillance of patients with lymphoblastic leukemia or lymphoma in remission. Blood 1981;58:214-20.
51. Buchanan GR, Smith RG. Detection of early relapse and minimal residual disease in children with acute lymphoblastic leukemia by enumeration of terminal deoxynucleotidyl transferase-positive cells in peripheral blood. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Boston: Martinus Nijhoff Publishers, 1984:83-95.
52. Bradstock KF, Janossy G, Hoffbrand AV, Ganeshaguru K, Llewellin P, Prentice HG, Bollum FJ. Immunofluorescent and biochemical studies of terminal deoxynucleotidyl transferase in treated acute leukaemia. Br J Haematol 1981;47:121-31.
53. Kalwinsky DK, Weatherred WH, Dahl GV, Bowman WP, Melvin SL, Coleman MS, Bollum FJ. Clinical utility of initial terminal deoxynucleotidyl transferase determinations in childhood acute leukemias. Cancer Res 1981;41:2877-81.
54. Hutton JJ, Coleman MS, Moffitt S, Greenwood MF, Holland P, Lampkin B, Kisker T, Krill C, Kastelic JE, Valdez L, Bollum FJ. Prognostic significance of terminal transferase activity in childhood acute lymphoblastic leukemia: a prospective analysis of 164 patients. Blood 1982;60:1267-76.
55. Barr RD, Koekebakker M, Sarin PS. Early relapse of acute lymphoblastic leukemia is not predictable by serial biochemical assays of terminal transferase activity in cells from peripheral blood. Leuk Res 1984;8:351-4.
56. Hooijkaas H, van Dongen JJM, Hählen K, van Zanen GE. Immunological characterisation of cells in cerebrospinal fluid from patients with lymphoid malignancies. Lancet 1984;i:518-9.
57. Hooijkaas H, van Dongen JJM, Hählen K, van Zanen GE. Identification and monitoring of terminal deoxynucleotidyl transferase positive cells in the cerebrospinal fluid of children with lymphoid malignancies. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1984:875-8.
58. Barr RD, Koekebakker M. Detection of circulating "terminal transferase-positive" cells does not predict relapse in acute lymphoblastic leukemia. Leuk Res 1984;8:1051-5.
59. Koekebakker M, Barr RD. Demonstration of terminal deoxynucleotidyl transferase in single cells by indirect immunofluorescence: a methodological reappraisal. Leuk Res 1983;7:237-41.
60. Bradstock KF, Papageorgiou ES, Janossy G, Hoffbrand AV, Willoughby ML, Roberts PD, Bollum FJ. Detection of leukaemic lymphoblasts in CSF by immunofluorescence for terminal transferase. Lancet 1980;i:1144.

61. Bradstock KF, Papageorgiou ES, Janossy G. Diagnosis of meningeal involvement in patients with acute lymphoblastic leukemia: Immunofluorescence for terminal transferase. Cancer 1981;47:2478-81.
62. Casper JT, Lauer SJ, Kirchner PA, Gottschall JL, Camitta BM. Evaluation of cerebrospinal fluid mononuclear cells obtained from children with acute lymphocytic leukemia: Advantages of combining cytomorphology and terminal deoxynucleotidyl transferase. Am J Clin Pathol 1983;80:666-70.
63. Greaves M, Delia D, Janossy G, Rapson N, Chessells J, Woods M, Prentice G. Acute lymphoblastic leukaemia associated antigen. IV. Expression on non-leukaemic "lymphoid" cells. Leuk Res 1980;4:15-32.
64. Greaves MF, Hariri G, Newman RA, Sutherland DR, Ritter MA, Ritz J. Selective expression of the common acute lymphoblastic leukemia (gp100) antigen on immature lymphoid cells and their malignant counterparts. Blood 1983;61:628-39.
65. Ryan DH, Mitchell SJ, Hennessy LA, Bauer KD, Horan PK, Cohen HJ. Improved detection of rare CALLA-positive cells in peripheral blood using multiparameter flow cytometry. J Immunol Methods 1984;74:115-28.
66. Braun MP, Martin PJ, Ledbetter JA, Hansen JA. Granulocytes and cultured human fibroblasts express common acute lymphoblastic leukemia-associated antigens. Blood 1983;61:718-25.
67. Neudorf SML, LeBien TW, Kersey JH. Characterization of thymocytes expressing the common acute lymphoblastic leukemia antigen. Leuk Res 1984;8:173-9.
68. Ritz J, Nadler LM, Bhan AK, Notis-McConarty J, Pesando JM, Schlossman SF. Expression of common acute lymphoblastic leukemia antigen (CALLA) by lymphomas of B-cell and T-cell lineage. Blood 1981;58:648-52.
69. Veerman AJP, Huismans DR, Van Zantwijk CH, De Waal FC. Immunologische bepaling van oppervlakte determinanten op cellen in de liquor cerebrospinalis van kinderen met acute lymfatische leukemie. Ned Tijdschr Geneeskd 1984;128:100-3.
70. Greaves M, Paxton A, Janossy G, Pain C, Johnson S, Lister TA. Acute lymphoblastic leukaemia associated antigen. III. Alterations in expression during treatment and in relapse. Leuk Res 1980;4:1-14.
71. McMichael AJ, Pilch JR, Galfré G, Mason DY, Fabre JW, Milstein C. A human thymocyte antigen defined by a hybrid myeloma monoclonal antibody. Eur J Immunol 1979;9:205-10.
72. McMillan EM, Brubaker DB, Peters S, Jackson I, Beeman K, Wasik R, Stoneking LE, Resler DR. Demonstration of cells bearing the OKT6 determinant in human tonsil and lymph node. Cancer Immunol Immunother 1983;15:221-6.
73. De Bruin HG, Astaldi A, Leupers T, van de Griend RJ, Dooren LJ, Schellekens PThA, Tanke HJ, Roos M, Vossen JM. T lymphocyte characteristics in bone marrow-transplanted patients. II. Analysis with monoclonal antibodies. J Immunol 1981;127:244-51.
74. Kluin-Nelemans HC, Bolhuis RLH, Löwenberg B, Campana D, Sizoo W. Characterization of normal and regenerating bone marrow cells with a panel of monoclonal antibodies. Scand J Haematol, in press.
75. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Boston: Martinus Nijhoff Publishers, 1984: 67-81.
76. Neudorf SML, LeBien TW, Bollum F, Kersey JH. Double fluorochrome analysis of human bone marrow lymphoid cells: studies with terminal transferase and anti-T-cell monoclonal anti-bodies. Exp Hematol 1984;12:69-73.
77. Bernard A, Raynal B, Lemerle F, Boumsell L. Changes in surface antigens on malignant T cells from lymphoblastic lymphomas at relapse: an appraisal with monoclonal antibodies and microfluorometry. Blood 1982;59:809-815.
78. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res 1981;5:301-9.
79. Donlon JA, Jaffe ES, Braylan RC. Terminal deoxynucleotidyl transferase activity in malignant lymphomas. N Engl J Med 1977;297:461-4.
80. Kung PC, Long JC, McCaffrey RP, Ratliff RL, Harrison TA, Baltimore D. Terminal deoxynucleotidyl transferase in the diagnosis of leukemia and malignant lymphoma. Am J Med 1978;64:788-94.
81. Braziel RM, Keneklis T, Donlon JA, Hsu SM, Cossman J, Bollum FJ, Jaffe ES. Terminal deoxynucleotidyl transferase in non-Hodgkin's lymphoma. Am J Clin Pathol 1983;80:655-9.

82. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
83. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J. Triple immunological staining with colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.
84. Krolick KA, Uhr JW, Vitetta ES. Selective killing of leukaemia cells by antibody-toxin conjugates: implications for autologous bone marrow transplantation. Nature 1982;295:604-5.
85. Hagenbeek A, Martens ACM. Detection of minimal residual disease in acute leukemia: possibilities and limitations. Eur J Cancer Clin Oncol 1985;21:389-95.
86. Bradstock KF, Kerr A. Immunological detection of covert leukaemic spread in mediastinal T-cell lymphoblastic lymphoma. Leuk Res 1985;9:905-11.
87. Weinstein HJ, Vance ZB, Jaffe N, Buell D, Cassady JR, Nathan DG. Improved prognosis for patients with mediastinal lymphoblastic lymphoma. Blood 1979;53:687-94.

CHAPTER 5.4

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE (TdT)-POSITIVE CELLS IN CEREBROSPINAL FLUID AND THE DEVELOPMENT OF OVERT CNS LEUKEMIA: A 5-YEAR FOLLOW-UP STUDY IN 113 CHILDREN WITH A TdT POSITIVE LEUKEMIA OR NON-HODGKIN LYMPHOMA*

Herbert Hooijkaas[1], Karel Hählen[2], Henk J. Adriaansen[1], Ina Dekker[2], George E. van Zanen[2], and Jacques J.M. van Dongen[1].

1. Department of Immunology, Erasmus University, University Hospital/Dijkzigt, Rotterdam;
2. Department of Pediatrics, Subdivision Hematology-Oncology, Sophia Children's Hospital, Rotterdam, The Netherlands.

SUMMARY

We investigated whether an indirect nuclear terminal deoxynucleotidyl transferase (TdT) immunofluorescence (IF) assay on single cells present in the cerebrospinal fluid (CSF) is more effective than conventional cytomorphology for early detection or exclusion of (minimal) meningeal leukemic infiltration in patients with a TdT^+ malignancy. During a 5-year follow-up study 1,661 consecutive CSF samples from 113 children with a TdT^+ acute lymphoblastic leukemia (ALL) (n=100), a TdT^+ acute non-lymphoblastic leukemia (ANLL) (n=8), or a TdT^+ non-Hodgkin lymphoma (NHL) (n=5) were analyzed. In 1,511 (91.9%) out of 1,643 evaluative CSF samples, the positive and negative findings of both cytomorphology and the TdT-IF assay were concordant. In 47 (2.9%) samples out of 28 patients the cytomorphology was suspect while the TdT-IF assay was negative; follow-up as long as 58 months revealed no central nervous system (CNS) leukemia in any patient. In 85 (5.2%) samples cytomorphology was negative (n=70) or suspect (n=15) but TdT^+ cells were detected. Red blood cell (RBC) contamination seriously hampered evaluation in 31 out of these 85 samples. From the remaining 54 TdT^+ samples out of 29 patients, 40 samples preceded overt CNS leukemia in 20 patients. Two consecutive findings of TdT^+ cells in the CSF were always followed by overt CNS leukemia. At initial diagnosis, 11 children had TdT^+ cells in their RBC-free CSF. In one of these children, morphology was suspect; a repeated lumbar puncture was positive on both assays. Thus, initial CNS leukemia was diagnosed. In the other 10 children, morphology was negative. In six of them, CNS leukemia was diagnosed 2 to 20 months later. In 32 other children examined at initial diagnosis, neither TdT^+ cells nor blasts were observed in the CSF. In none of these

Published in: Blood 1989;74:416-22.

patients was a CNS leukemia diagnosed after a follow-up of 2.5 to 57 months (median 24 months). In 207 control CSF samples from 58 children with TdT^- oncologic, hematologic or infectious diseases, no TdT^+ cells could be detected. The TdT-IF assay is easy to perform and is a more reliable diagnostic tool for the detection of CNS leukemia at an early stage than cytomorphology. At initial diagnosis, the finding of TdT^+ cells in a RBC-free CSF sample with a negative cytomorphology is highly predictive for development of overt CNS leukemia.

INTRODUCTION

Extramedullary disease, in particular central nervous system (CNS) involvement, still represents a major concern in acute lymphoblastic leukemia (ALL) and T cell non-Hodgkin lymphoma (NHL) in childhood (1). As a result of improved systemic therapy, the growth of tumor cells in the CNS has become apparent (1,2). Prevention as well as treatment of early CNS involvement are important goals of therapy. In 2 to 15% of patients, relapses occur primarily in the CNS (2-16), notwithstanding CNS "prophylaxis". Early diagnosis of CNS leukemia implies unequivocal detection of a minimal number of leukemic blasts in the CSF. Such early detection of CNS leukemia has definite consequences for the choice of therapy, although one should be aware that it represents a rather late stage of leukemic infiltration of the meninges (17). In many protocols for management of patients with leukemia or NHL, cell counting and cytomorphologic examination of the CSF at regular intervals have been integrated. Diagnostic problems arise when the CSF cell count is low and cytomorphology is suggestive but not conclusive for the presence of malignant cells (18-22). Viral infections or chemotherapy can change normal mononuclear cells into atypical blastlike cells (22-24). Furthermore, malignant lymphoblasts may have the appearance of relatively well-differentiated lymphocytes (25). Therefore, the sensitivity and specificity of classic cytomorphologic techniques for detection of malignant lymphoid cells is insufficient and improvements can be expected, e.g. by use of immunologic marker analysis of CSF cells. Such analysis can be performed with monoclonal antibodies (McAb) or conventional antisera specific for cell surface antigens (18,20,26-32) or for the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) (25,33-37). TdT is present on the nuclear membrane of immature lymphoid cells of both B cell and T cell lineages and of their malignant counterparts. All ALL (except the infrequently occurring mature B-ALL) and some T-NHL, especially lymphoblastic lymphomas, are TdT^+ (38,39). TdT is also expressed in ~20% of ANLL (40-42). Normally a substantial proportion of the mononuclear bone marrow (BM) cells (up to 11%) and thymocytes may be TdT^+. Only low percentages of TdT^+ cells (<0.4%) have been detected at other sites, such as peripheral blood (PB) and lymph nodes (39,43,44). However, no TdT^+ cells have been detected in the CSF from individuals not suffering from leukemia or NHL (25,39). Thus, the presence of TdT^+ cells in the CSF indicates the presence of malignant cells. For the detection of small numbers of TdT^+ cells, the biochemical TdT assay is insensitive as compared with the TdT immunofluorescence (TdT-IF) assay (45,46). In addition, the TdT-IF assay combined with phase contrast morphology is preferable to cell-surface antigen analysis with McAb for routine analysis of CSF for the presence of lymphoblasts, since no single specific cell-surface marker covers all subtypes of ALL and some subtypes of ANLL and NHL as efficiently as does TdT.

To determine whether the TdT-IF assay improves the detection or exclusion of (minimum) leukemic CNS infiltrates in patients with TdT^+ malignancies, a longitudinal study was started in 1983. We report a 5-year follow-up study involving 1,661 CSF samples from 113 children with TdT^+ ALL (n=100), TdT^+ ANLL (n=8), or TdT^+ NHL (n=5).

MATERIALS AND METHODS

Patients and evaluation of data

From February 1, 1983 till February 1, 1988, all children with a newly diagnosed TdT^+ leukemia (n=98) or TdT^+ NHL (n=5) entered the study. In addition, all children with a cytomorphologically and clinically proven meningeal involvement diagnosed before February 1, 1983 (n=10) were included. A total number of 1,661 CSF samples from 113 patients was analyzed. As a control group, 207 CSF samples from 58 children with TdT^- leukemia, TdT^- NHL, or other hematologic, oncologic, or infectious diseases were analyzed.

The diagnosis of CNS leukemia was made on cytomorphologic criteria only, irrespective of the CSF cell count. Two successive CSF samples were examined for this purpose; the second sample was taken at the start of intrathecal therapy. A conclusion was reached after blast cells had been judged by at least two independent observers. Since the TdT-IF assay was considered to be experimental, treatment for CNS involvement was never instituted on the basis of the presence of TdT^+ cells in the CSF alone. The cytomorphologic investigations were performed in the diagnostic laboratory of the Subdivision Hematology/Oncology and the TdT-IF assay was performed in the diagnostic laboratory of the Department of Immunology.

All children were treated according to current local protocols or to those of the Dutch Childhood Leukemia Study Group (DCLSG). Since 1984, a spinal tap was performed at initial diagnosis (without intrathecal therapy). According to protocol, every 3 months a bone marrow (BM) puncture and a spinal tap were performed, until 4 to 5 years after initial diagnosis.

For evaluation of the predictive value of the TdT-IF assay for development of CNS leukemia, only patients who did not relapse at other sites were considered. For calculation of the median follow-up, patients were censored at the moment of relapse at other sites.

Treatment protocols

Four treatment protocols were used related to clinical characteristics at diagnosis. The children without high-risk characteristics (i.e. WBC $<50 \times 10^9$/l, no mediastinal enlargement, no initial CNS leukemia) were treated according to national DCLSG protocol V, in use until November 1983 (A)(47), or protocol VI, in use from November 1983 to July 1988 (B)(48). Children with an initial WBC $>50 \times 10^9$/l were treated according to a local protocol, in use until December 1987 (C). Children with mediastinal involvement and with >25% blasts or without a leukemic BM were treated with a modified APO protocol (D).

Induction treatment in protocols A, B and C consisted of vincristine (VCR), prednisone, and L-asparaginase. A randomization for addition of daunorubicin was made in protocol A. Maintenance treatment consisted of 7-wk cycles of 5 wk of daily oral 6-mercaptopurine (6-MP) and weekly methotrexate (MTX) alternating with 2 wk of prednisone and 2 weekly injections of VCR for 2 years (A,B) or 3 years (C). In protocol B, prednisone was substituted by dexamethasone from December 1984 on. In protocol C, cyclophosphamide, once every fortnight, was added in the 6-MP/MTX period during the first y of maintenance treatment. In protocol D, induction treatment consisted of VCR, prednisone, and doxorubicin, followed by L-asparaginase. Maintenance treatment in this protocol consisted of pulses of high-dose prednisone, high-dose 6-MP, VCR, and doxorubicin every 3 wk; five days of MTX was substituted for doxorubicin after the first 9 mo (49,50).

CNS prophylaxis for protocols A and C consisted of 25 Gy craniocervical irradiation plus five intrathecal injections of MTX (delivered in 2,5 wk) as soon as remission-induction treatment ended. In protocol C, six intrathecal injections with MTX, with a 7-wk interval, were administered in addition during the first y of maintenance

treatment.

In protocol B, CNS prophylaxis consisted of two intrathecal injections of MTX at wk 2 and 4 of remission-induction treatment, followed by three 24-hour infusions of 2 g MTX plus an intrathecal injection of MTX, delivered weekly during the first 3 wk after the end of remission-induction treatment. During the first y of treatment, triple (MTX, cytarabine (ARA-C), and prednisone) therapy was administered intrathecally every 7 wk (eight times) (48). In protocol D, the originally described CNS prophylaxis (50) with cranial irradiation plus five intrathecal injections of MTX followed by a intrathecal injection of MTX once every 18 wk was changed as follows: CNS prophylaxis consisted of three intrathecal injections of MTX plus ARA-C at wk 3, 6, and 9 during remission-induction and consolidation treatment, followed by three 24-hour infusions, once every fortnight, of 2 g MTX plus intrathecal MTX and ARA-C followed by an intrathecal injection of MTX plus ARA-C every 6 wk during 1 y (eight times).

Cell sampling and cytomorphology

CSF was obtained by lumbar puncture and collected in two plastic tubes, 2 to 5 ml each. One tube was processed in the diagnostic laboratory of the Subdivision of Hematology/Oncology within 30 minutes after sampling for cell counting and cytomorphology; the other was sent to the diagnostic laboratory of the Department of Immunology and was subjected to the TdT-IF assay within three hours after sampling. Cells were counted in a Fuchs-Rosenthal chamber. At least two cytocentrifuge preparations were made (0.5 ml each) with a Shandon-Elliott centrifuge (72 g, ten minutes) and stained with May-Grunwald-Giemsma or additional cytochemical stainings. Cytomorphology was considered positive when blasts were found that were unequivocally considered leukemic, irrespective of the CSF cell count. CSF samples that contained questionable blastlike cells or atypical (nonviral) lymphocytes were regarded as "suspect".

TdT-IF assay

For the TdT-IF assay, CSF samples (2 to 5 ml) were centrifuged (300 g, 5 minutes). The cell pellets were resuspended to 100 μl and two cytocentrifuge preparations were made (200 g, 3 minutes, Cytofuge, Nordic Immunological Laboratories, Tilburg, The Netherlands). These were air-dried, fixed in methanol (30 minutes, 4°C) and washed in phosphate-buffered saline (PBS), pH 7.8, for 15 minutes. One preparation was incubated with 15 μl optimally titrated rabbit anti-TdT antiserum; the second preparation, as a control, was incubated with 15 μl normal rabbit serum (moist chamber, 30 minutes, room temperature). After being washed in PBS, the preparations were incubated with 15 μl fluorescein conjugated goat anti-rabbit immunoglobulin antiserum and washed again (51). The anti-TdT antiserum and the second-step antiserum were obtained from Bethesda Research Laboratories (Bethesda, MD) and from Supertechs (Bethesda, MD). The preparations were then mounted in glycerol PBS (9:1) containing *p*-phenylenediamine 1 mg/ml (BDH Chemicals, Poole, UK) to prevent fading of fluorochrome, covered with a coverslip and sealed with paraffin wax with ceresin (BDH chemicals) (52). Cells expressing nuclear TdT were enumerated using Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, FRG) equipped with phase contrast facilities (52). A concentration effect (centrifugation of 2 to 5 ml CSF) might be the reason why we almost invariably detected cells on the TdT cytocentrifuge preparations, even when cell counts were low ($<5/\mu$l). If possible 100 to 2,000 cells were screened. In 19% of cases, however, low cell count of the CSF allowed screening of 10 to 100 cells only; 63% of the preparation contained 100 to 500 cells, whereas 18% contained <10 cells. The percentage of TdT^+ cells was calculated as the fraction of the number of nucleated cells, as determined by phase contrast morphology. If any TdT^+ cells were found in an atraumatic (RBC-free) spinal tap, the result of the TdT-IF assay was considered positive, irrespective of the total number of cells in cytocentrifuge preparation or the CSF count.

RESULTS

Of 1,661 CSF samples available, 18 samples were non- evaluative for comparison between cytomorphology and the TdT-IF assay since it proved impossible to perform both examinations.

In 1,643 samples from 113 children with a TdT$^+$ acute leukemia or NHL the correlation between cytomorphology and the TdT-IF assay could be examined. Findings are summarized in Table 1. In 88.0% of the 1,643 evaluative samples, both cytomorphology and the TdT-IF assay were negative; in 3.9% of the 1,643 samples, the results were both positive. The cytomorphology was suspect in 62 samples (3.8%). In 47 out of these samples (2.9%) from 28 patients no TdT$^+$ cells were observed. No meningeal involvement has occurred in these patients during a median follow-up period of 26 months (range: 1 to 58 months). In the remaining 15 (0.9%) cytomorphologically suspect samples TdT$^+$ cells were observed. In 70 other samples (4.3%) in which no blasts were observed by cytomorphologic examination, TdT$^+$ cells proved to be present (Table 1).

The follow-up of the 49 ALL, one ANLL, and two NHL patients who contributed to the 85 TdT$^+$ but cytomorphologically suspect or negative CSF samples is summarized in Tables 2 and 3. Table 2 shows data of children with TdT$^+$ samples at initial diagnosis; Table 3 shows data of those with a first TdT$^+$ sample during maintenance therapy or after cessation of treatment. Data are subdivided in CSF samples with or without RBC contamination (Tables 2 and 3). The 31 RBC-contaminated CSF samples (18 at diagnosis and 13 during follow-up) were considered non-assessable. Out the 54 RBC-free TdT$^+$ samples, 11 were obtained at initial diagnosis from nine ALL and two T-NHL patients (Table 2). The percentages of TdT$^+$ cells ranged from 0.3 to 70% (median 13%). In one child the CSF was cytomorphologically suspect, and a spinal tap repeated after 5 days contained both TdT$^+$ cells and leukemic blasts according to cytomorphologic criteria. Thus, initial CNS leukemia was diagnosed. In the other ten patients a second CSF sample obtained 2 to 3 weeks later, during the systemic induction treatment, did not contain TdT$^+$ cells anymore. However, six out of ten patients (five ALL and one T-NHL) developed a CNS leukemia as diagnosed by cytomorphology 2 to 20 months later. The other four patients did not develop a CNS leukemia during a median follow-up of 38 months (range 31 to 55 months). In 32 patients without TdT$^+$ cells in their RBC-free CSF samples at diagnosis, no CNS leukemia was diagnosed during a median follow-up of 24 months (range 2.5 to 57 months).

The other 43 RBC-free TdT$^+$ CSF samples from 17 ALL, one ANLL and two T-NHL patients were obtained during follow-up on or off therapy (Table 3). Nine patients (ten samples) did not develop CNS leukemia; five out of them (six samples), however, relapsed in the BM and accor-

TABLE 1. Correlation between cytomorphology and TdT-IF assay in 1,643 CSF samples from 113 patients with a TdT$^+$ ALL (n = 100), a TdT$^+$ ANLL (n = 8), or a TdT$^+$ NHL (n=5).

	TdT-IF assay		
Cytomorphology	Negative	Positive	Total
Negative	1,446 (88.0%)	70 (4.3%)	1,516 (92.3%)
Suspect	47 (2.9%)	15 (0.9%)	62 (3.8%)
Positive	0 (0%)	65 (3.9%)	65 (3.9%)
Total	1,493 (90.9%)	150 (9.1%)	1,643 (100.0%)

TABLE 2. Follow-up of 27 ALL and two NHL patients with TdT^+ cells in CSF at initial diagnosis, but with negative or suspect CSF cytomorphology (n = 29 samples).

									CSF TdT-IF assay		Clinical course	
CSF sample	Patient No.	Sex	Age (y)	WBC at Diagnosis ($x10^9$/l)	PB Blasts (%)	BM Blasts (%)	Immunologic Phenotype	Treatment Protocol[a]	TdT^+ Cells	Inter-pretetion	CNS Leukemia	Follow-up (mo)
No RBC, negative cytomorphology	1	M	7	62.0	75	83	T-ALL	D	0.3	+	no	42+
	2	M	2	34.9	62	95	common ALL	B	5.0	+	yes	12
	3	M	3	10.3	37	98	common ALL	B	11	+	yes	2
	4	F	3	14.5	58	93	pre-B ALL	B	13	+	no	31+
	5	M	2	63.0	91	100	common ALL	C	14	+	no	34+
	6	M	15	3.1	60	68	common ALL	A	15	+	yes	17
	7	F	5	266.0	73	92	T-ALL	D	18	+	yes	6
	8	F	4	1.5	27	85	pre-B ALL	B	70	+	yes	20
	9	M	13	7.0	0	1	T-NHL	D	2	+	no	55+
	10	F	8	9.9	0	3	T-NHL	D	2	+	yes	3
No RBC, suspect cytomorphology	11	M	2	5.5	59	85	common ALL		55	+	initial	–
RBC contaminated, negative or suspect cytomorphology	12	M	12	124.4	90	97	T-ALL	D	43	NA[b]	yes	0.5
	13-29	F:5	2-13	3.0-402.0	7-99	79-99	common ALL:9	B:12	0.2-67	NA	no	5.5-37+
	(n = 17)	M:12	(m:5)[c]	(m:19.4)	(m:59)	(m:95)	pre-B ALL:4	C:3				(m:20)[d]
							T-ALL:4	D:2				

a. Treatment protocols A, B, C, and D are described in the Materials and Methods section.
b. NA, not assessable due to RBC contamination.
c. m, median.
d. Three patients developed a BM relapse.

TABLE 3. Evaluation of 29 patients with TdT+ cells in CSF during follow-up but with a negative or suspect CSF cytomorphology (n = 56 samples).

			TdT-IF assay		Clinical course	
RBC in CSF	Number of patients[a]	Number of samples	TdT+ cells (%)	Interpretation	Relapsed in the CNS	Follow-up in mo Median (range)
no	13	33	0.1-72	positive	yes	2 (0.5-24)
no	9	10	0.2-3.0	positive	no[b]	4 (1-48+)
yes	12	13	0.01-1.4	not assessable[c]	no[d]	7 (4-56+)

a. Four patients are represented in more than one category.
b. Five patients developed a BM relapse.
c. Three patients developed a BM relapse.
d. Not assessable due to RBC contamination.

dingly received reinduction therapy, including CNS prophylaxis. Two samples from two patients were obtained in the last phase of successful CNS leukemia reinduction treatment and represented the last positive finding in a series of several TdT+ samples in which cytomorphology was already negative. One of the remaining two samples was positive during CNS prophylaxis, and the other was positive during maintenance therapy. These two patients did not develop CNS leukemia at 48 and 46 months of follow-up, respectively. In 13 patients (33 samples) findings of TdT+ cells in the CSF were followed by a diagnosis of overt CNS leukemia within a median follow-up of 1.5 months (range 0.5 to 24 months). In our series, two consecutive findings of TdT+ cells in the CSF were always followed by overt CNS leukemia.

TdT+ cells were never detected in 207 RBC-free CSF samples from 58 patients with TdT− leukemia or NHL or with other hematologic, oncologic, or infectious diseases. Moreover, several patients in our leukemia and NHL follow-up study had episodes with bacterial or viral meningitis, sometimes paralleled with high CSF cell counts. However, neither were TdT+ cells noted in the CSF of patients with these infections.

DISCUSSION

The present study was undertaken to investigate (a) whether the presence of TdT+ cells in the CSF, as demonstrated by TdT-IF assay, would be helpful for early diagnosis of CNS leukemia and (b) whether the absence of TdT+ cells would strongly argue in favor of the absence of meningeal involvement. The result would have direct implications for treatment.

Our data indicate that all CNS leukemias diagnosed on clinical and cytomorphologic criteria were always confirmed by TdT-IF assay, resulting in a 100% sensitivity. In addition, we were able to detect TdT+ cells before overt CNS leukemia according to cytomorphologic criteria could be diagnosed (Tables 2 and 3). We further investigated whether the presence of TdT+ cells (even in low numbers) is indicative for meningeal involvement. TdT+ cells do not belong to the normal CSF cell population and, as a consequence, every single TdT+ cell in the CSF should be considered malignant (25,35). Our experience concurs with these assumptions (36,39,45). The present study confirms the findings of other investigators (25) that TdT+ cells

do not occur in the CSF of non-leukemic patients. We further investigated the CSF of patients with a TdT^+ malignancy who received systemic treatment or prophylactic CNS treatment which may prevent development of CNS leukemia.

Each finding of TdT^+ cells does not appear to be followed by overt CNS leukemia (Tables 2 and 3). This may be a consequence of treatment. During sampling the CSF may also become contaminated at times with leukemic or non-leukemic TdT^+ cells from PB (23,53,54) or even BM (47,55-57). Such contamination may occur especially at diagnosis, when high numbers of TdT^+ tumor cells can be present in PB and BM. Therefore, at diagnosis as well as during follow-up simultaneous analysis of both CSF and PB for TdT^+ cells is desirable. However, during traumatic lumbar puncture only ~20% of the predicted numbers of WBC are found in the CSF, suggesting that a direct extrapolation of PB data to data of contaminated CSF is not accurate (8-60). We and other investigators believe that CSF samples with leukemic (or TdT^+) cells derived from such contaminated punctures cannot be evaluated properly (22,23,53). In such cases the spinal tap should be repeated. For this reason, we have regarded results of TdT^+ RBC-containing CSF samples as nonassessable (Tables 2 and 3).

Forty-three children had RBC-free CSF samples at initial diagnosis. In 32 of them no TdT^+ cells were present in the CSF. In none of them was overt CNS leukemia diagnosed during a median follow-up of 24 months (range 2.5 to 57 months). Eleven of the 43 patients had TdT^+ cells in their CSF. In one of them TdT^+ cells were present while cytomorphology was suspect; initial CNS leukemia was unequivocally diagnosed at a repeated spinal tap five days later. In the other ten patients, no blasts were noted on cytomorphologic examination. Thus, based on the TdT-IF assay alone, the incidence of detectable CNS involvement at diagnosis was 25% (11 out of 43), much higher than the commonly reported 5% based on cytomorphologic criteria (1,14). Indeed, six out of ten children (60%) not diagnosed as having initial CNS leukemia, eventually developed overt CNS leukemia despite CNS prophylaxis. Why in the four patients with 0.3 to 14% TdT^+ cells in the CSF at diagnosis overt CNS leukemia was not diagnosed after a follow-up of 31 to 55 months is not clear. All ten children with TdT^+ cells in the CSF at initial diagnosis received comparable CNS prophylaxis. At present, we must assume that the CNS prophylaxis was able to prevent or eliminate meningeal leukemia in these four patients. However, CNS leukemia can still occur many years after initial diagnosis (6); thus, the period of follow-up may still be too short. Nevertheless, our data indicate that a positive TdT-IF assay in the first RBC-free CSF sample of untreated patients selects for those at high risk of developing overt CNS leukemia according to standard criteria. Thus, we believe that the finding of TdT^+ cells in a RBC-free CSF sample at diagnosis should be taken into account for a diagnosis of initial CNS leukemia and as a consequence should lead to a more intensive CNS treatment.

Concerning the patients under maintenance treatment or treated for CNS leukemia, two consecutive findings of TdT^+ cells in RBC-free CSF samples without cytomorphologically detectable leukemic blasts were always followed by a cytomorphologically confirmed CNS leukemia. Incidental findings of TdT^+ cells in the CSF from such patients did not always result in overt CNS leukemia as a first event. Therapy, especially the therapy administered as a consequence of relapses at other sites than the CNS, might have prevented the development of overt meningeal leukemia in these patients. However, a first finding of TdT^+ cells in the CSF from patients off therapy was always followed by a second finding. Subsequently, overt CNS

leukemia always resulted, indicating that a single finding of TdT$^+$ cells in a CSF sample from patients after cessation of treatment might well be sufficient for the diagnosis of CNS leukemia relapse.

In our experience and that of other researchers the TdT-IF assay can be performed easily, even when low numbers of cells are present in the CSF (25,35). The TdT-IF assay proved highly sensitive for evaluation of CSF samples from patients with TdT$^+$ leukemia or NHL. This assay contributes to early detection as well as exclusion of CNS leukemia. In our opinion, it is the most reliable tool for diagnosing CNS leukemia in patients with TdT$^+$ malignancies. Moreover, it also offers the possibility for selecting diagnosis of patients at high risk of developing overt CNS leukemia. Therefore, the diagnostic criteria for presence or absence of CNS leukemia should include the findings of the TdT-IF assay.

ACKNOWLEDGMENTS. We thank Prof. Dr. R. Benner for his continuous support, the medical and nursing staff as well as all laboratory personnel for their skilful contributions, Dr. A. Hagemeijer, Dr. A.C. Knulst, Dr. J.P. van de Merwe for critically reading the manuscript, and Geertje de Korte for expert secretarial assistance.

This work was supported by the Netherlands Cancer Foundation.

REFERENCES

1. Anonymous. Leukaemia and the central nervous system (Editorial). Lancet 1985;i:1196-8.
2. Evans AE, Gilbert ES, Zandstra R. The increasing incidence of central nervous system leukemia in children. (Children's cancer study group A). Cancer 1970;26:404-9.
3. Aur RJA, Simone J, Hustu HO, Walters T, Borella L, Pratt C, Pinkel D. Central nervous system therapy and combination chemotherapy of childhood lymphocytic leukemia. Blood 1971;37:272-81.
4. Aur RJA, Simone JV, Hustu HO, Verzosa MS. A comparative study of central nervous system irradiation and intensive chemotherapy early in remission of childhood acute lymphocytic leukemia. Cancer 1972;29:381-91.
5. Hustu HO, Aur RJA, Verzosa MS, Simone JV, Pinkel D. Prevention of central nervous system leukemia by irradiation. Cancer 1973;32:585-97.
6. Komp DM, Fernandez CH, Falletta JM, Ragab AH, Humphrey GB, Pullen J, Moon T, Shuster J. CNS prophylaxis in acute lymphoblastic leukemia. Comparison of two methods. A Southwest oncology group study. Cancer 1982;50:1031-6.
7. Nesbit ME, D'Angio GJ, Sather HN, Robinson LL, Ortega J, Donaldson M, Hammond GD. Effect of isolated central nervous system leukaemia on bone marrow remission and survival in childhood acute lymphoblastic leukaemia. A report for children's cancer study group. Lancet 1981;i:1386-9.
8. Sullivan MP, Chen T, Dyment PG, Hvizdala E, Steuber CP. Equivalence of intrathecal chemotherapy and radiotherapy as central nervous system prophylaxis in children with acute lymphatic leukemia: a pediatric oncology group study. Blood 1982;60:948-58.
9. Freeman AI, Weinberg V, Brecher ML, Jones B, Glicksman AS, Sinks LF, Weil M, Pleuss H, Hananian J, Burgert EO Jr, Gilchrist GS, Necheles T, Harris M, Kung F, Patterson RB, Maurer H, Leventhal B, Chevalier L, Forman E, Holland JF. Comparison of intermediate-dose methotrexate with cranial irradiation for the post-induction treatment of acute lymphocytic leukemia in children. N Engl J Med 1983;308:477-84.
10. Inati A, Sallan SE, Cassady JR, Hitchcock-Bryan S, Clavell LA, Belli JA, Sollee N. Efficiacy and morbidity of central nervous system 'prophylaxis' in childhood acute lymphoblastic leukemia: eight years' experience with cranial irradiation and intrathecal methotrexate. Blood 1983;61:297-303.
11. Anderson JR, Wilson JF, Jenkin RDT, Meadows AT, Kersey J, Chilate RR, Coccia P, Exelby P. Childhood non-Hodgkin's lymphoma. The results of a randomized therapeutic trial comparing a 4-drug regimen (COMP) with a 10-drug regimen (LSA_2-L_2). N Engl J Med 1983;308:559-65.

12. Sackmann Muriel F, Svarch E, Pavlovsky S, Eppinger-Helft M, Braier J, Vergara B, Gary G, Kvicala R, Divito JM, Failace R, Dibar E, Jimenez E. Comparison of central nervous system prophylaxis with cranial radiation and intrathecal methotrexate versus intrathecal methotrexate alone in acute lymphoblastic leukemia. Blood 1983;62:241-50.
13. Gasparini M, Lattuada A, Lombardi F, Rilke F, Gianni C, Fossati-Bellani F. Childhood non-Hodgkin's lymphoma: prognostic relevance of clinical stages and histologic subgroups. Am J Pediatr Hematol Oncol 1983;5:161-71.
14. Lampert F, Henze G, Langermann H-J, Schellong G, Gadner H, Riehm H-J. Acute lymphoblastic leukemia: current status of therapy in children. In: Thiel E, Thierfelder S, eds. Recent results in cancer research. Berlin: Springer-Verlag, 1984;93:159-81.
15. Pinkerton CR, Chessells JM. Failed central nervous system prophylaxis in children with acute lymphoblastic leukemia: treatment and outcome. Br J Haematol 1984;57:553-61.
16. Thompson CB, Sanders JE, Flournoy N, Buckner CD, Thomas ED. The risks of central nervous system relapse and leukoencephalopathy in patients receiving marrow transplants for acute leukemia. Blood 1986;67:195-9.
17. Price RA, Johnson WW. The central nervous system in childhood leukemia: I. The arachnoid. Cancer 1973;31:520-33.
18. Bigner SH, Johnston WW. The cytopathology of cerebrospinal fluid. I. Nonneoplastic conditions, lymphoma and leukemia. Acta Cytol 1981;25:335-53.
19. Borowitz M, Bigner SH, Johnston WW. Diagnostic problems in the cytologic evaluation of cerebrospinal fluid for lymphoma and leukaemia. Acta Cytol 1981;25:665-74.
20. Magennis H, Markey G, Alexander HD, Morris TCM. Leukaemia/lymphoma cells in cerebrospinal fluid. J Clin Pathol 1983;36:606.
21. An-Foraker SH. Cytodiagnosis of malignant lesions in cerebrospinal fluid. Review and cytohistologic correlation. Acta Cytol 1985;29:286-90.
22. McIntosh S, Ritchey AK. Diagnostic problems in cerebrospinal fluid of children with lymphoid malignancies. Am J Pediatr Hematol Oncol 1986;8:28-31.
23. Woodruff KH. Cerebrospinal fluid cytomorphology using cytocentrifugation. Am J Clin Pathol 1973;60:621-7.
24. Choi H-S, Anderson PJ. Diagnostic cytology of cerebrospinal fluid by the cytocentrifuge method. Am J Clin Pathol 1979;72:931-43.
25. Bradstock KF, Papageorgiou ES, Janossy G. Diagnosis of meningeal involvement in patients with acute lymphoblastic leukemia: immunofluorescence for terminal transferase. Cancer 1981;47:2478-81.
26. Martin SE, Zhang H-Z, Magyarosy E, Jaffe ES, Hsu S-M, Chu EW. Immunologic methods in cytology: definitive diagnosis of non-Hodgkin's lymphomas using immunologic markers for T- and B-cells. Am J Clin Pathol 1984;82:666-73.
27. Hohlfeld R, Brüske-Hohlfeld I, Schwartz A, Brocke U, Toyka KV. Analyse von Oberflächenmarkern auf Liquorzellen. Beitrag zur Differentialdiagnostik intrathekaler Lymphome. Dtsch Med Wochenschr 1984;109:1760-2.
28. Veerman AJP, Huismans LDR, van Zantwijk ICH. Diagnosis of meningeal leukemia using immunoperoxidase methods to demonstrate common acute lymphoblastic leukemia cells in cerebrospinal fluid. Leuk Res 1985;9:1195-1200.
29. Homans AC, Forman EN, Barker BE. Use of monoclonal antibodies to identify cerebrospinal fluid lymphoblasts in children with acute lymphoblastic leukemia. Blood 1985;66:1321-5.
30. Li C-Y, Witzig TE, Phyliky RL, Ziesmer SC, Yam LT. Diagnosis of B-cell non-Hodgkin's lymphoma of the central nervous system by immunocytochemical analysis of cerebrospinal fluid lymphocytes. Cancer 1986;57:737-44.
31. Vick WW, Wikstrand CJ, Bullard DE, Kemshead J, Coakham HB, Schlom J, Johnston WW, Bigner DD, Bigner SH. The use of a panel of monoclonal antibodies in the evaluation of cytologic specimens from the central nervous system. Acta Cytol 1987;31:815-24.
32. Yam LT, English MC, Janckila AJ, Ziesmer S, Li CY. Immunocytochemistry of cerebrospinal fluid. Acta Cytol 1987;31:825-33.
33. Bradstock KF, Papageorgiou ES, Janossy G. Detection of leukaemic lymphoblasts in CSF by immunofluores-

cence for terminal transferase. Lancet 1980;i:1144.
34. Peiper S, Stass SA, Bollum FJ. Detection of leukaemic lymphoblasts in CSF. Lancet 1980;ii:39.
35. Casper JT, Lauer SJ, Kirchner PA, Gottschall JL, Camitta BM. Evaluation of cerebrospinal fluid mononuclear cells obtained from children with acute lymphocytic leukemia: advantages of combining cytomorphology and terminal deoxynucleotidyl transferase. Am J Clin Pathol 1983;80:666-70.
36. Hooijkaas H, van Dongen JJM, Hählen K, van Zanen GE. Immunological characterisation of cells in cerebrospinal fluid from patients with lymphoid malignancies. Lancet 1984;i:518-9.
37. Hooijkaas H, van Dongen JJM, Hählen K, van Zanen GE. Identification and monitoring of terminal deoxynucleotidyl transferase positive cells in the cerebrospinal fluid of children with lymphoid malignancies. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1984:875-8.
38. Braziel RM, Keneklis T, Donlon JA, Hsu S-M, Cossman J, Bollum FJ, Jaffe ES. Terminal deoxynucleotidyl transferase in non-Hodgkin's lymphoma. Am J Clin Pathol 1983;80:655-9.
39. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: Martinus Nijhoff Publishers, 1986:113-33.
40. Bradstock KF, Hoffbrand AV, Ganeshaguru K. Terminal deoxynucleotidyl transferase expression in acute non-lymphoid leukaemia: an analysis by immunofluorescence. Br J Haematol 1981;47:133-43.
41. Cuttner J, Seremetis S, Najfeld V, Dimitriu-Bona A, Winchester RA. TdT-positive acute leukemia with monocytoid characteristics: clinical, cytochemical, cytogenetic and immunologic findings. Blood 1984;64:237-43.
42. Greaves MF, Chan LC, Furley AJW, Watt SM, Molgaard HV. Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 1986;67:1-11.
43. Froehlich TW, Buchanan GR, Cornet JAM, Sartain PA, Smith RG. Terminal deoxynucleotidyl transferase-containing cells in peripheral blood: implications for the surveillance of patients with lymphoblastic leukemia or lymphoma in remission. Blood 1981;58:214-20.
44. Bradstock KF, Kerr A, Bollum FJ. Antigenic phenotype of TdT-positive cells in human peripheral blood. Cell Immunol 1985;90:590-8.
45. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, van der Linde-Preesman AA, Tettero ILM, van de Rijn M, Hilgers J, van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukaemia. Dordrecht: Martinus Nijhoff Publishers, 1984:67-81.
46. Bollum FJ. Terminal deoxynucleotidyl transferase as a hematopoietic cell marker. Blood 1979;54:1203-15.
47. Van der Does-van den Berg A, Van Wering ER, De Koning J, Rammeloo JA, Solbu G, Suciu S, Van Zanen GE. Addition of rubidomycine to induction treatment with vincristine, prednisone and L-asparaginase in standard-risk acute lymphocytic leukemia (Study ALL V): a report on behalf of the Dutch Childhood Leukemia Study Group. Haematol Blood Transf 1987;30:444-7.
48. Veerman AJP, Hählen K, Kamps WA, Van Leeuwen EF, De Vaan GAM, Van Wering ER, Van der Does-van den Berg A, Solbu G, Suciu S. Dexamethasone, medium dose methotrexate and prolonged intrathecal therapy in the treatment of standard risk ALL; preliminary results of the DCLSG study ALL VI. Med Pediatr Oncol 1988;16:396.
49. Weinstein HJ, Link MP. Non-Hodgkin's lymphoma in childhood. Clin Haematol 1979;8:699-716.
50. Weinstein HJ, Cassady JR, Levey R. Long-term results of the APO protocol (vincristine, doxorubicin (Adriamycin) and prednisone) for treatment of mediastinal lymphoblastic lymphoma. J Clin Oncol 1983;1:537-54.
51. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, de Klein A, van Zanen GE, van't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50
52. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological characterization of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GF, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: Martinus Nijhoff Publishers, 1987:87-116.

53. Komp DM. Diagnosis of CNS leukemia. Am J Pediatr Hematol Oncol 1979;1:31-5.
54. Rohlfing MB, Barton TK, Bigner SH, Johnston WW. Contamination of cerebrospinal fluid specimens with hematogenous blasts in patients with leukemia. Acta Cytol 1981;25:611-5.
55. Kruskall MS, Carter SR, Ritz LP. Contamination of cerebrospinal fluid by vertebral bone-marrow cells during lumbar puncture. N Engl J Med 1983;308:697-700.
56. Lane PA, Githens JH. Contamination of cerebrospinal fluid with bone-marrow cells during lumbar puncture. N Engl J Med 1983;309:434-5.
57. Luban NLC, Alessi RM, Gold BG, Kapur S. Cerebral spinal fluid pleocytosis with bone marrow contamination. J Pediatr 1984;104:254-6.
58. Chow G, Schmidley JW. Lysis of erythrocytes and leukocytes in traumatic lumbar punctures. Arch Neurol 1984;41:1084-5.
59. Novak RW. Lack of validity of standard corrections for white blood cell counts of blood-contaminated cerebrospinal fluid in infants. Am J Clin Pathol 1984;82:95-7.
60. Rubenstein JS, Yogev R. What represents pleocytosis in blood-contaminated ('traumatic tap') cerebrospinal fluid in children? J Pediatr 1985;107:249-51.

CHAPTER 6

CLINICAL APPLICATIONS OF IMMUNOGLOBULIN GENE AND T CELL RECEPTOR GENE ANALYSIS

CHAPTER 6.1

INTRODUCTION: IMMUNOGLOBULIN AND T CELL RECEPTOR GENES IN CLONAL EXPANSIONS OF LYMPHOCYTES

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

During the last decade the classification of lymphoproliferative diseases and related disorders has been improved by the application of immunologic marker analysis (1-7). Generally this is sufficient for identifying the B or T cell origin of a lymphoid malignancy and often allows a more detailed classification with the recognition of subgroups of the disease (1-7). The application of immunologic marker analysis for diagnostic purposes is discussed extensively in Chapter 5.

It is also possible to prove the clonal origin of mature B cell malignancies which express immunoglobulin (Ig) molecules, by demonstrating single Ig light chain expression (10-14). Since clonality is equivalent to malignancy in the majority of cases, this test is useful for the distinction between malignant and reactive B cell populations (1,2,5,10-14). However, immature B cell malignancies, such as acute lymphoblastic leukemia (ALL) of the B cell type, and several mature B cell malignancies do not express Ig molecules and in case of T cell malignancies there is no appropriate marker for clonality at the protein level (1,2,4-10). Many hematopoietic malignancies have chromosome aberrations (15-17) which can be used as markers for clonality and often also as tumor-specific markers (17,18). However, optimal cytogenetic analysis can only be performed on freshly isolated cell samples or tissue biopsies and even then it may be difficult to obtain metaphases of the malignant cells (15,19).

In some lymphoproliferative disorders the distinction between a malignant and a reactive process and the determination of the lineage association of the malignancy may be difficult or impossible on the basis of morphological studies and immunologic marker analysis. In these cases clonality and the origin of the involved lymphoid cell populations may be determined by studying the configuration of the genes which code for the antigen-specific receptors of lymphocytes: the Ig genes and the T cell receptor (TcR) genes (20-25). This approach is based on the fact that B and T cells rearrange their Ig and TcR genes, respectively, and that these gene rearrangements are different in each lymphocyte, but identical within a lymphocyte clone. This holds for both physiological and malignant clonal expansion. The configuration of germline and (clonally) rearranged Ig and TcR genes can be studied by Southern blot analysis (see Chapter 3.3).

In Chapter 6.2 we shall summarize the occurrence of Ig and TcR gene rearrangements in

the various types of leukemias and non-Hodgkin lymphomas (NHL) to demonstrate that leukemias and NHL can at least partly be used as model system for studying the hierarchic order of Ig and TcR gene rearrangements during normal lymphoid differentiation. In Chapter 6.3 we shall critically discuss the possibilities and limitations of Ig and TcR gene analysis for diagnosis and management of lymphoproliferative diseases and related disorders. This will be illustrated by a series of clinical cases.

REFERENCES

1. Tubbs RR, Fishleder A, Weiss RA, Savage RA, Sebek BA, Weick JK. Immunohistologic cellular phenotypes of lymphoproliferative disorders: comprehensive evaluation of 564 cases including 257 non-Hodgkin's lymphomas classified by the international working formulation. Am J Pathol 1983;113:207-21.
2. Doggett RS, Wood GS, Horning S, Levy R, Dorfman RF, Bindl J, Warnke RA. The immunologic characterization of 95 nodal and extranodal diffuse large cell lymphomas in 89 patients. Am J Pathol 1984;115:245-52.
3. Chan LC, Pegram SM, Greaves MF. Contribution of immunophenotype to the classification and differential diagnosis of acute leukaemia. Lancet 1985;i:475-9.
4. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
5. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987;128:181-201.
6. Schuurman H-J, Van Baarlen J, Huppes W, Lam BW, Verdonck LF, Van Unnik JAM. Immunophenotyping of non-Hodgkin's lymphoma: lack of correlation between immuno-phenotype and cell morphology. Am J Pathol 1987;129:140-51.
7. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.
8. McMichael AJ, Beverly PCL, Cobbold S, Crumpton ML, Gilks W, Gotch FM, Hogg N, Horton M, Ling N, MacLennan ICM, Mason DY, Milstein C, Spiegelhalter D, Waldmann H, eds. Leucocyte typing III: white cell differentiation antigens. Oxford: Oxford University Press, 1987:1050.
9. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: M Nijhoff, 1987:87-116.
10. Fialkow PJ, Klein E, Klein G, Clifford P, Singh S. Immunoglobulin and glucose-6-phosphate dehydrogenase as markers of cellular origin in Burkitt lymphoma. J Exp Med 1973;138:89-102.
11. Levy R, Warnke R, Dorfman RF, Haimovich J. The monoclonality of human B-cell lymphomas. J Exp Med 1977;145:1014-28.
12. Teodorescu M, Mayer EP. Surface immunoglobulin in immunoproliferative diseases. Ann Clin Lab Sci 1978;8:353-65.
13. Ault KA. Detection of small numbers of monoclonal B lymphocytes in the blood of patients with lymphoma. N Engl J Med 1979;300:1401-5.
14. Ligler FS, Smith RG, Kettman JR, Hernandez JA, Himes JB, Vitetta ES, Uhr JW, Frenkel EP. Detection of tumor cells in the peripheral blood of nonleukemic patients with B-cell lymphoma: analysis of "clonal excess". Blood 1980;55:792-801.
15. Rowley JD. Do all leukemic cells have an abnormal karyotype? N Engl J Med 1981;305:164-6.
16. Yunis JJ, Bloomfield CD, Ensrud K. All patients with acute nonlymphocytic leukemia may have a chromosomal defect. N Engl J Med 1981;305:135-9.
17. Yunis JJ. The chromosomal basis of human neoplasia. Science 1983;221:227-36.
18. Rowley JD. Biological implications of consistent chromosome rearrangements in leukemia and lymphoma. Cancer Res 1984;44:3159-68.
19. Harrison C, ed. Association of clinical cytogeneticists. A guide to cytogenetic studies in haematological disorders. London: Eugenics Society Occasional Papers, second series no.1,1987:27.
20. O'Connor NTJ, Gatter KC, Wainscoat JS, Crick J, Jones DB, Delsol G, Ralfkiaer E, De Wolf-Peeters C, Angus

B, Mason DY. Practical value of genotypic analysis for diagnosing lymphoproliferative disorders. J Clin Pathol 1987;40:147-50.

21. Korsmeyer SJ. Antigen receptor genes as molecular markers of lymphoid neoplasms. J Clin Invest 1987;79:1291-5.
22. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.
23. Sklar J, Weiss LM. Applications of antigen receptor gene rearrangements to the diagnosis and characterization of lymphoid neoplasms. Ann Rev Med 1988;39:315-34.
24. Cossman J, Uppenkamp M, Sundeen J, Coupland R, Raffeld M. Molecular genetics and the diagnosis of lymphoma. Arch Pathol Lab Med 1988;112:117-27.
25. Knowles DM. Immunophenotypic and antigen receptor gene rearrangement analysis in T cell neoplasia. Am J Pathol 1989;134:761-85.

CHAPTER 6.2

REARRANGEMENT OF IMMUNOGLOBULIN AND T CELL RECEPTOR GENES IN LEUKEMIAS AND NON-HODGKIN LYMPHOMAS*

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/
University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

It has widely been accepted that each type of leukemia and non-Hodgkin lymphoma (NHL) has a normal cellular counterpart and that each of these counterparts can be fitted into a normal differentiation/maturation pathway (1-5). Several lymphoid malignancies indeed have obvious cellular counterparts with identical morphology and immunophenotype. However, this concept remains an "unproven hypothesis", since for several malignancies a normal counterpart has not (yet) been found (6-9).

Some reports emphasize the diagnostic usefulness of immunophenotypic differences between lymphoid malignancies and their "normal counterparts" (7,9,10). These differences include both acquisition and loss of immunologic markers (7,9,10). Discrepancies between morphological and immunologic classifications of hematopoietic malignancies also occur, especially in acute myeloid leukemias (AML) and NHL (7-11). However, these discrepancies are at least partly due to the frequent occurrence of subpopulations, which may have different phenotypes (5).

Despite the mentioned discrepancies, hypothetical differentiation schemes as presented in Chapters 2.3 and 5.1 form a useful conceptual framework for the relationship between the various lymphoid malignancies. It also allows the evaluation of the possible relationship between the differentiation stage of malignancies and the configuration of their Immunoglobulin (Ig) and T cell receptor (TcR) genes.

IMMUNOGLOBULIN GENE REARRANGEMENTS IN B CELL MALIGNANCIES

The various types of precursor B cell acute lymphoblastic leukemia (precursor B-ALL; i.e. null ALL, common ALL and pre-B-ALL) and B-ALL can be regarded as malignant counterparts of immature B cells (1-5). The chronic B cell leukemias, such as B-CLL, B cell prolymphocytic leukemia (B-PLL) and hairy cell leukemia (HCL) as well as the various types of B-NHL can be

---

This manuscript will be published as part of a review in Clin Chim Acta.

TABLE 1. IgH and IgL gene analysis of nineteen precursor B-ALL and seven chronic B cell leukemias.

Patient no.	Type of leukemia[a]	IgH genes[b] JH probe		Igκ genes[b] Cκ probe	Igλ genes[b] Cλ probe	Igλ genes[b] λ-IVS probe
		*Bgl*II	*Eco*RI	*Bam*HI	*Eco*RI	*Eco*RI
1.	null ALL	G/G	G/G	G/G	G/G	8.3/8.3[c]
2.	null ALL	R/R/G^w	R/R/G^w	G/G	G/G	8.3/8.3
3.	null ALL[d]	R/R^w/R^w/G [e]	R/G	G/G	G/G	8.3/8.3
4.	null ALL	R/R	R/R	G/G	G/G	8.3/8.3
5.	null ALL	R/R/G^w	R/R/G^w	G/G	G/G	8.3/19.1
6.	common ALL	R/G^w	R/G^w	G/G	G/G	8.3/13.7
7.	common ALL	R/G^w	R/G^w	G/G	G/G	8.3/8.3
8.	common ALL[d]	R/R/R/G^w [e]	R/R/G^w	G/G	G/G	8.3/8.3
9.	common ALL	R/R	R/R	G/G	G/G	8.3/8.3
10.	common ALL[d]	R/R/R^w [e]	R/R/R	R/G	G/G	8.3/24.5
11.	common ALL[d]	R/R/R^w/R^w/G	R/R/R/R^w/G	R/G	G/G	8.3/8.3
12.	common ALL[d]	R/R/R/G^e	R/R/R/G	R/G	R/G	8.3/10[g]
13.	common ALL	R/G	R/G	G/G	R/R	8.3/–[h]
14.	pre-B-ALL	R/G	R/R	G/G	G/G	8.3/19.1
15.	pre-B-ALL[d]	R/R^w/R^w/R^w [e]	R/R	G/G	G/G	8.3/8.3
16.	pre-B-ALL	R/R	R/R/R	G/G	G/G	8.3/8.3
17.	pre-B-ALL[d]	R/R/R/R/R^w/R^w	R/R/R/R [e]	G/G	G/G	8.3/19.1
18.	Smκ$^+$ B-ALL	R/R	R/R	D/R	G/G	8.3/8.3
19.	Smλ$^+$ B-ALL	R/R	R/R	D/D	R/R	–/–[h]
20.	Smκ$^+$ B-CLL	R/R	R/R	D/R	G/G	8.3/8.3
21.	Smκ$^+$ B-CLL	R/G	R/G	D/R	G/G	8.3/19.1
22.	Smκ$^+$ B-CLL	R/R/G	R/R/G	R/G	G/G	8.3/8.3
23.	Smλ$^+$ B-CLL	R/G	R/R	D/D	R/R	13.7/–[h]
24.	Smλ$^+$ B-CLL	R/R	R/R^w/R^w [f]	D/D	D/R	–[h]/10[g]
25.	Smλ$^+$ B-CLL	R/R^w/R^w/G [f]	R/R/G	D/D	R/R	8.3/5[g]
26.	Smκ$^+$ B-PLL	R/R	R/R	D/R	G/G	8.3/8.3

a. The different types of precursor B-ALL are defined as follows: null ALL: CD19$^+$, CD10$^-$, weak Cyμ$^-$, SmIg$^-$; common ALL: CD19$^+$, CD10$^+$, weak Cyμ$^-$, SmIg$^-$; pre-B-ALL: CD19$^+$, CD10$^+$, weak Cyμ$^+$, SmIg$^-$; B-ALL: CD19$^+$, CD10$^{+ \text{ or } -}$, weak Cyμ$^-$, SmIg$^+$ (see ref.5).
b. G, germline band; G^w, weak germline band (Probably caused by contaminating non-B cells); R, rearranged band; R^w, weak rearranged band; D, deletion of the involved gene (segment).
c. The numbers represent the sizes (in kb) of the bands detected with the λ-IVS probe in *Eco*RI digests.
d. In patients 3, 8, 10, 11, 12, 15 and 17 more than two rearranged bands were found in at least two different digests.
e. These patterns of multiple rearranged bands were confirmed in *Bam*HI/*Hin*dIII digests.
f. In patients 24 and 25 one strong rearranged band and two weak rearranged bands were detected in one digest. This was not confirmed in other digests.
g. The 5 kb and 10 kb bands detected with the λ-IVS probe in *Eco*RI digests probably represent non-germline bands due to rearrangements involving the Cλ2-Cλ3 gene segment.
h. In some leukemias the Cλ2-Cλ3 gene segment is probably deleted on one or both alleles.

regarded as the counterparts of B cells in mature stages of differentiation (2,3,5). Based on this concept the various B cell malignancies have repeatedly been used to study the Ig heavy chain (IgH) and Ig light chain (IgL) gene rearrangements during lymphoid differentiation (12-28). As an example of this type of approach we have summarized the Southern blot data of twenty-five leukemias of B cell lineage in Table 1 and Figures 1-3. Data from several reports on

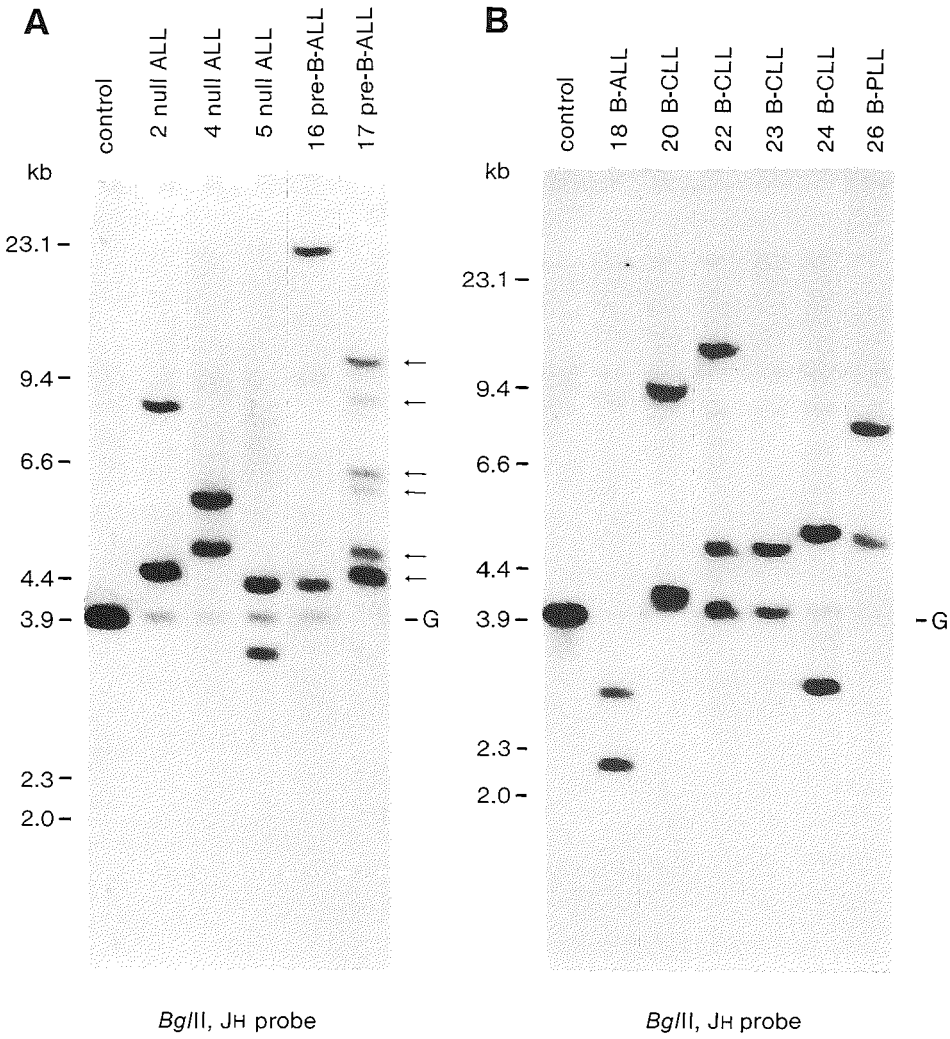


Figure 1. IgH gene analysis of precursor B-ALL, B-ALL and chronic B cell leukemias (see Table 1). DNA samples from control cells and from the leukemic cells were digested with *Bgl*II, size separated and blotted to a nylon membrane, which was hybridized with a ^{32}P-labeled JH probe. **A**: precursor B-ALL; **B**: SmIg$^+$ B cell leukemias. In most leukemias two rearranged bands were detected, but in some precursor B-ALL (e.g. patient 17) multiple rearranged bands were found.

large series of leukemias are summarized in Table 2 (12-26).

Except for a minority of precursor B-ALL (2%), especially null ALL, all leukemias of B cell origin have rearranged their IgH genes (Tables 1 and 2). In most of them rearrangement and/or deletion has occurred on *both* IgH gene alleles: 80 to 85% of ALL and 75 to 80% of chronic B cell leukemias (13,17,18,20-24; Table 1). It has been suggested that monoallelic IgH gene rearrangements occur more frequently in Smκ^+ than in Smλ^+ malignancies (26). Rearrange-

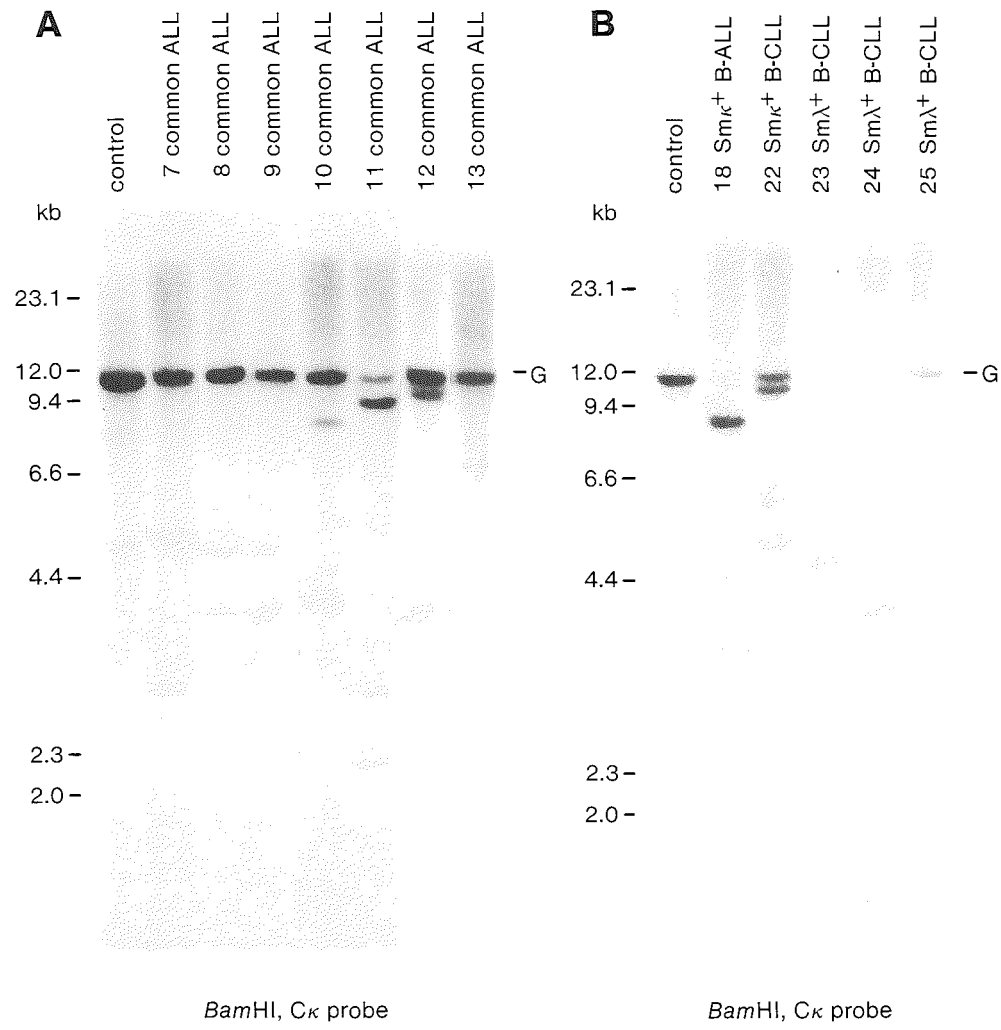


Figure 2. Igκ gene analysis of precursor B-ALL, B-ALL and chronic B cell leukemias (see Table 1). DNA samples from control cells and from the leukemic cells were digested with *Bam*HI, size separated and blotted to a nylon membrane, which was hybridized with a ^{32}P-labeled Cκ probe. **A**: precursor B-ALL; **B**: SmIg$^+$ B cell leukemias. In a few precursor B-ALL Igκ gene rearrangements were detected. All Smκ$^+$ B cell leukemias had rearranged Igκ genes, while the Cκ gene segment appeared to be deleted on both alleles in all Smλ$^+$ B cell leukemias.

ments and/or deletions in Igκ and Igλ occur in 5 to 25% of precursor B-ALL. However, no clear hierarchic pattern of IgL gene rearrangements was found, since they appear to occur in all three types of precursor B-ALL in variable frequencies (Table 2).

The pattern of IgL gene rearrangements is more clear in chronic B cell leukemias. All Smκ$^+$ leukemias had rearranged one or both Igκ genes, while no Igλ gene rearrangements or biallelic Igκ gene deletions were found (Tables 1 and 2). All Igλ$^+$ B cell leukemias had rearranged at least one Igλ gene allele and virtually all of them had deleted and/or rearranged their Igκ genes.

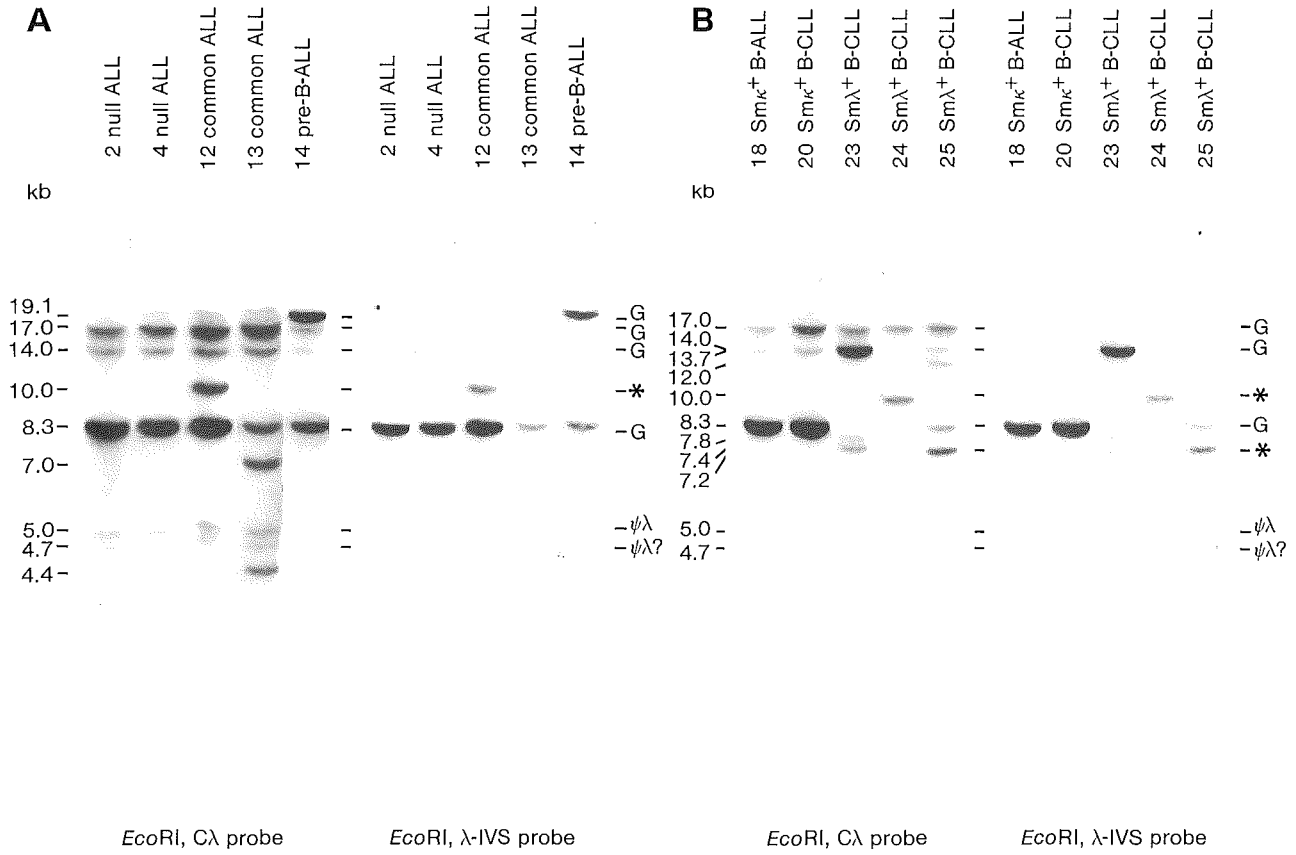


Figure 3. Igλ gene analysis of precursor B-ALL, B-ALL and chronic B cell leukemias (see Table 1). DNA samples from control cells and from the leukemic cells were digested with *Eco*RI, size separated and blotted to a nylon membrane, which was hybridized with a ^{32}P-labeled Cλ probe (**A**) and subsequently rehybridized with a ^{32}P-labeled λ-IVS probe (**B**). A few precursor B-ALL and all Smλ$^+$ B cell leukemias had rearranged Igλ genes. The 8.3 kb, 13.7 kb and 24.5 kb bands detected with the λ-IVS probe represent germline bands of normal or amplified Cλ2-Cλ3 gene segments; the 5 kb and 10 kb bands represent non-germline bands due to rearrangements involving the Cλ2-Cλ3 gene segment.

TABLE 2. Ig gene rearrangements in leukemias of B cell lineage, as reported in the literature.

	Rearranged IgH genes (R/G or R/R)	Rearranged Igκ genes (R/G, R/R or R/D)	Deleted Igκ genes (D/D or D/G)	Rearranged Igλ genes (R/G or R/R)
Acute lymphoblastic B cell leukemias[a]				
null ALL ($CD10^-$, $Cy\mu^-$)	95% (52/55)	13% (5/38)	8% (3/38)	9% (2/23)[b]
common ALL ($CD10^+$, $Cy\mu^-$)	99% (119/120)	32% (22/69)	12% (8/69)	2% (1/52)
pre-B-ALL ($CD10^+$, $Cy\mu^+$)	100% (33/33)	8% (1/12)	8% (1/12)	20% (2/10)
all $CD10^+$ precursor B-ALL[c]	98% (193/196)	23% (28/121)	12% (14/121)	5% (5/92)
all precursor B-ALL	98% (245/251)	21% (33/159)	11% (17/159)	6% (7/115)
B-ALL ($SmIg^+$)	100% (3/3)			
Chronic B cell leukemias[d]				
$Sm\kappa^+$ B-CLL	100% (27/27)	100% (18/18)	0% (0/18)	0% (0/19)
$Sm\kappa^+$ B-PLL	100% (8/8)	100% (6/6)	0% (0/6)	0% (0/4)
$Sm\kappa^+$ HCL	100% (12/12)	100% (12/12)	0% (0/12)	0% (0/12)
$Sm\lambda^+$ B-CLL	100% (18/18)	7% (1/14)	86% (12/14)	100% (11/11)[b]
$Sm\lambda^+$ B-PLL	100% (7/7)	50% (3/6)	50% (3/6)	100% (5/5)
$Sm\lambda^+$ HCL	100% (17/17)	12% (2/17)	83% (10/12)[e]	100% (16/16)

a. The data concerning the ALL of B cell origin are derived from references 12-22.
b. In one null ALL, a $Sm\lambda^+$ B-CLL and a few $Sm\lambda^+$ HCL rearranged Igλ genes were found in the presence of germline Igκ genes (ref. 17,24 and 25).
c. The subgroup "$CD10^+$ precursor B-ALL" include common ALL ($CD10^+$, $Cy\mu^-$) and pre-B-ALL ($CD10^+$, $Cy\mu^+$) as well as the $CD10^+$ precursor B-ALL (a total of 43 patients) from publications which did not report $Cy\mu$ staining.
d. The data concerning the chronic B cell leukemias are derived from refs. 23-26.
e. In several cases of HCL the presence of non-leukemic cells hampered the detection of κ deletions as determined by use of the Cκ probe (23,25). These cases were excluded from the table.

Only a few leukemias with rearranged Igλ genes but germline Igκ genes have been reported (17,24,25 and ALL patient 13 in Table 1). In 75 to 80% of leukemias with rearranged Igλ genes, the Cκ gene segment appears to be deleted on both alleles (12,17,18,21,23,24,26).

Few studies on IgH and IgL gene analysis in B-NHL have been reported. It appears that in B-NHL the IgH and IgL gene rearrangements are comparable to those in chronic B cell malignancies (27,28).

These data together indicate that a hierarchy in Ig gene rearrangements exists, which nevertheless seems to be less obvious than predicted from the combined murine and human data on normal B cell differentiation (c.f. Figure 11 in Chapter 3.2). This especially concerns the IgL gene rearrangements in precursor B-ALL, since Igκ and/or Igλ gene rearrangements have been found in all three types of precursor B-ALL (Table 2). From the leukemia data it can be concluded that IgH genes rearrange earlier than IgL genes, that Igκ genes generally rearrange prior to Igλ genes, and that in most cases with Igλ gene rearrangement Igκ gene segments have been deleted on both alleles (Figure 4).

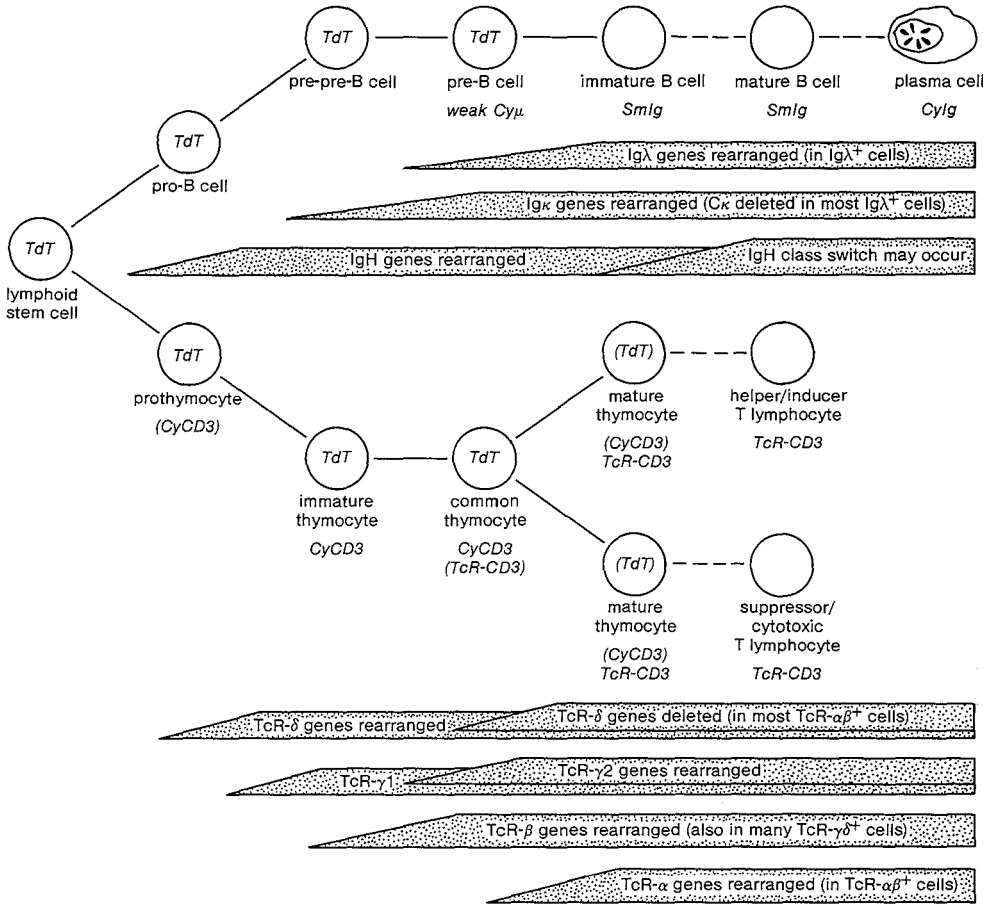


Figure 4. Hypothetical scheme of human lymphoid differentiation, summarizing the available data about the ordered rearrangement of Ig and TcR genes as determined by the analysis of leukemias and NHL (c.f. Figure 11 in Chapter 3.2).

T CELL RECEPTOR GENE REARRANGEMENTS IN T CELL MALIGNANCIES

The T lymphoblastic malignancies such as T-ALL and lymphoblastic lymphomas of T cell origin can be regarded as malignant counterparts of cells in immature T cell differentiation stages. About 65 to 70% of these immature T cell malignancies do not express a TcR-CD3 complex on their cell membrane ($CD3^-$ T cell malignancies), while 30 to 35% are $CD3^+$ (31), which implies that they express a TcR on the membrane (32,33). The mature T cell malignancies include the various types of peripheral T cell lymphomas, the T cell prolymphocytic leukemias (T-PLL) and the human T cell leukemia virus 1 (HTLV-I) positive adult T cell leukemia-lymphomas (ATLL) as well as the cutaneous T cell leukemia-lymphomas (CTLL) and related diseases such as Sézary syndrome (SS) (c.f. Figure 1 in Chapter 5.1). The far majority

of these mature T cell malignancies express CD3. The heterogeneous group of the more benign T cell proliferations such as T-CLL, T-γ lymphocytosis and large granular lymphocyte (LGL) proliferations will be discussed in Chapter 6.3.

In studies on the rearrangement and expression of TcR genes during human T cell differentiation, generally T-ALL have been used (19,21,22,34-60). In most studies only TcR-β genes and/or TcR-γ genes have been analyzed (19,21,22,34-52). Recently, also data about the TcR-δ genes have been reported (54-60), while appropriate data about TcR-α gene rearrangements are still scarce (53,61,62) due to the complexity of the TcR-α gene locus with the long stretch of Jα gene segments (61-65). Therefore, analysis of the TcR-α gene locus needs multiple Jα probes (53,61,62,66-68), which have to be distributed throughout the complete Jα region in order to detect each TcR-α gene rearrangement (53,61,62). Data from a large series of reports about TcR gene analysis of T cell malignancies (34-60,69-75) are summarized in Table 3. Data about T cell lines are not included in this table (61,62,67).

Approximately 85% of the $CD3^-$ lymphoblastic T cell malignancies have rearranged their TcR-γ and TcR-β genes (Figure 5 and Table 3). Several $CD3^-$ T-ALL (45,57,76,77) and $CD3^-$ T-NHL (78,79) have rearranged TcR-γ genes and germline TcR-β genes, but also a few $CD3^-$ T cell leukemias with germline TcR-γ genes and rearranged TcR-β genes have been reported (21,46). In some of the latter cases TcR-γ gene rearrangements may have been missed, because only restricted TcR-γ gene analyses were performed.

In the far majority of $CD3^+$ T cell malignancies both TcR-β and TcR-γ gene rearrangements have been found (Figure 5 and Table 3). Only four of the $CD3^+$ T-ALL and three of the $CD3^+$ T-NHL in Table 3 had germline TcR-β genes (21,37,48,57). In three of the four T-ALL cases it was proven that the leukemic cells expressed TcR-γδ (21,55,57). Probably also other reported $CD3^+$ T cell malignancies with germline TcR-β genes express TcR-γδ (49,56,78,80-82). However, in most TcR-$\gamma\delta^+$ T-ALL the TcR-β genes are rearranged (57,59).

In most T cell malignancies (86 out of 98 tested; i.e. 88%) the TcR-β gene rearrangements involved the β2 locus on one or both alleles (21,34,38,40,41,46,47,49,50,82). This would imply that analysis of the β2 locus detects the majority of TcR-β gene rearrangements in T cell malignancies. The TcR-γ gene rearrangements in T cell malignancies involve the γ2 locus on both alleles in 77% (66/86) of cases, while the remaining 23% have monoallelic or biallelic rearrangements within the γ1 locus (49,51,52,59,71,82).

Recently, data about TcR-δ genes in T cell malignancies have become available (54-60). These reports indicate that in virtually all $CD3^-$ and $CD3^+$ T cell malignancies the TcR-δ genes are rearranged and/or deleted (Figure 5 and Table 3). Even six immature T-ALL cases with germline TcR-β and TcR-γ genes appeared to have rearranged TcR-δ genes (55-57). Most TcR-δ gene rearrangements appeared to involve the Jδ1 gene segment (41 out of 47 rearranged TcR-δ gene alleles; i.e. 87%), while a few rearrangements to the Jδ2 gene segment were found (13%) (54,56,59). Rearrangements in the TcR-α genes are infrequent in $CD3^-$ T cell malignancies, but occur in high frequencies in $CD3^+$ T cell malignancies (Table 3) (53,54). Rearrangement and/or transcription of the TcR-α genes generally correspond with deletion of the TcR-δ genes (54,55,57,59), which is in line with the fact that the TcR-δ genes are located within the TcR-α gene locus between the Vα and Jα gene segments (61,62,65).

The majority (~80%) of TcR-$\alpha\beta^+$ T cell malignancies have deleted both TcR-δ gene alleles (31,54,55,57,59) but in some of them (~20%) one germline or rearranged TcR-δ gene allele is

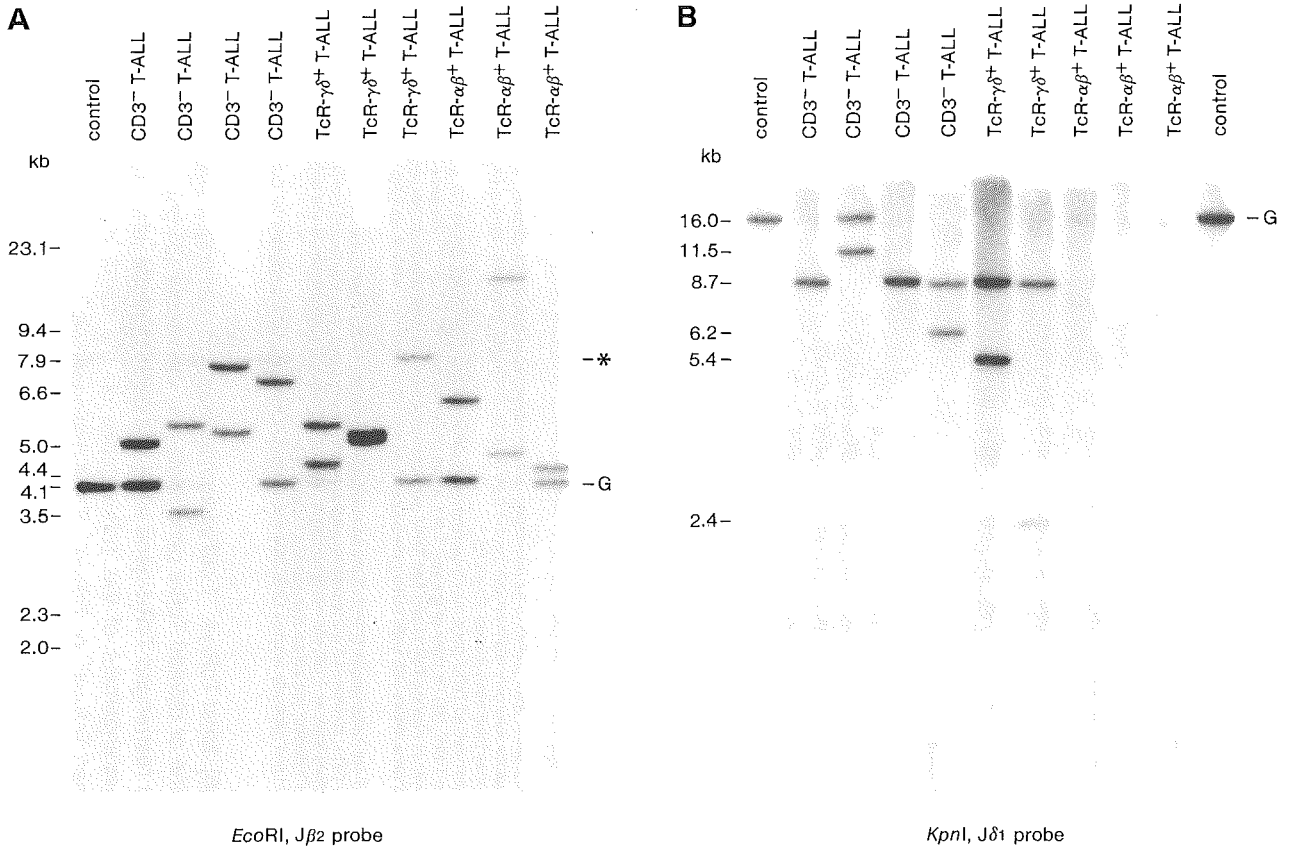


Figure 5. Analysis of the TcR-β genes and TcR-δ genes of a series of T-ALL. DNA samples from control cells and from the T-ALL cell samples were digested with *Eco*RI or *Kpn*I, size separated and blotted to nylon membrane filters. **A**: *Eco*RI filter hybridized with a ^{32}P-labeled Jβ2 probe. **B**: *Kpn*I filter hybridized with a ^{32}P-labeled Jδ1 probe. Both the CD3$^-$ T-ALL and the CD3-TcR$^+$ T-ALL had rearranged TcR-β genes. The weak 7.9 kb band, present in some of the lanes, is due to partial digestion (c.f. Figure 16 in Chapter 3.3). The TcR-δ genes were rearranged in all CD3$^-$ T-ALL as well as the TcR-$\gamma\delta^+$ T-ALL, but the TcR-δ genes were deleted on both alleles in the TcR-$\alpha\beta^+$ T-ALL.

TABLE 3. TcR gene rearrangements in various types of T cell malignancies, as reported in the literature[a].

	Rearranged TcR-α genes[b] (R/G or R/R)	Rearranged TcR-β genes (R/G or R/R)	Rearranged TcR-γ genes (R/G or R/R)	Rearranged TcR-δ genes (R/G, R/R or R/D)	Deleted TcR-δ genes (D/D or D/G)
Lymphoblastic T cell malignancies[c]					
$CD3^-$ T-ALL	14% (1/7)[d]	84% (144/172)	85% (106/124)[e]	76% (48/63)	16% (10/63)
$CD3^+$ T-ALL	67% (8/12)	96% (105/109)[f]	100% (85/85)	58% (31/53)[g]	42% (22/53)
all T-ALL		89% (249/281)	91% (191/209)	68% (79/116)	28% (32/116)
T-NHL		79% (11/14)[f]	100% (2/2)		
Mature T cell malignancies[h]					
T-PLL		100% (22/22)	100% (7/7)	0% (0/9)	100% (9/9)
ATLL[i]		100% (36/36)	95% (21/22)[e]		
peripheral T-NHL		93% (25/27)[f]	100% (12/12)		
Lennert lymphoma		100% (5/5)	100% (5/5)		
CTLL/MF		100% (27/27)			
SS		100% (22/22)	100% (2/2)		

a. Many reports on TcR gene rearrangements in a series of T cell malignancies have been published (34-60,69-75). Several research groups have published more than once data on a series of T cell malignancies, which may therefore be (partly) identical. To avoid double counting, we have only included the most informative data of each research group by selecting the publications: refs. 53 and 54, leaving out refs. 35,40,51 and 68; combination of refs. 21 and 55; combination of refs. 49 and 56; ref. 74, leaving out refs. 73 and 75.

b. Data on TcR-α gene rearrangements in T cell leukemias were only included if sufficient Jα probes were used. Therefore only refs. 53 and 54 were used, while refs. 66 and 68 were excluded.

c. The lymphoblastic T cell malignancies include T-ALL and lymphoblastic T-NHL. The data are derived from refs. 19,21,22,27,34,36-39,41-44,46-50,53-60,74.

d. In one $CD3^-$ T-ALL the TcR-α genes were proven to be rearranged on both alleles, while both TcR-δ gene alleles were deleted (53,54).

e. In some studies the TcR-γ genes were only analyzed with the Jγ1.3 probe in *Eco*RI digests. Therefore rearrangements to the Jγ1.1, Jγ1.2 and Jγ2.1 gene segments may have been missed (21,46,72).

f. In seven $CD3^+$ T cell malignancies no TcR-β gene rearrangements were detected: four $CD3^+$ T-ALL (21,48,57), one $CD3^+$ lymphoblastic T-NHL (37) and two mature T-NHL (37). In three of the four T-ALL it was proven to concern a TcR-$\gamma\delta^+$ leukemia (21,55,57).

g. Nine $CD3^+$ T-ALL were proven to express TcR-γδ and had TcR-δ gene rearrangements on both alleles (57,59). Another four $CD3^+$ T-ALL had rearranged both TcR-δ gene alleles with both TcR-α gene alleles in germline configuration, while three $CD3^+$ T-ALL had TcR-δ(D/R) and TcR-α(R/G) (54).

h. The data about the mature T cell malignancies (T-NHL, T cell non-Hodgkin lymphoma; T-PLL, T cell prolymphocytic leukemia; ATLL, adult T cell leukemia-lymphoma; CTLL, cutaneous T cell leukemia-lymphoma; SS, Sézary Syndrome) are derived from refs.: 27,35-39,41,49,69-72. Chronic T cell proliferations such as T cell chronic lymphocytic leukemia (T-CLL), T-γ lymphocytosis and large granular lymphocyte (LGL) proliferations are not included, but will be discussed in Chapter 6.3.

i. ATLL are positive for HTLV-I and generally express CD3, CD4 and CD25 (IL-2 receptor), but are negative for CD8 (72).

still present (31,55,56). Most TcR-$\gamma\delta^+$ T cell leukemias have two rearranged TcR-δ gene alleles, although one germline or deleted TcR-δ gene allele may also occur (31,56). This implies that the detection of one rearranged TcR-δ gene allele and deletion of the other TcR-δ gene allele (with a possible rearrangement of the TcR-α genes on the same allele) does not allow discrimination between TcR-$\alpha\beta^+$ or TcR-$\gamma\delta^+$ T cell malignancies.

All data together suggest that the TcR-δ genes rearrange in the early stages of T cell differentiation. Subsequently the TcR-γ genes rearrange (γ1 probably before γ2), immediately followed by rearrangement of the TcR-β genes. In a part of the cells the TcR-α genes rearrange together with or after deletion of the TcR-δ genes (Figure 4). This results in TcR-$\gamma\delta^+$ cells

(TcR-γ: R/R or R/G; TcR-δ: R/G, R/R or R/D; TcR-β: R or sometimes G/G) or in TcR-$\alpha\beta^+$ cells (TcR-α: R/R or R/G; TcR-β: R/R or R/G; TcR-γ: R/R or R/G; TcR-δ: D/D or sometimes D/R).

Based on our experience and data from the literature, we estimate that 60 to 75% of the $CD3^+$ T cell malignancies express TcR-$\alpha\beta$, while 25 to 40% express TcR-$\gamma\delta$. However, further studies with appropriate monoclonal antibodies (see Appendix of Chapter 2.2) (31) have to determine the precise frequencies of these phenotypes and have to reveal whether these frequencies are dependent on the type of T cell malignancy.

LINEAGE INFIDELITY OF Ig AND TcR GENE REARRANGEMENTS

Initially it was assumed that many immunologic, enzymatic and molecular characteristics of B and T cells were lineage specific or even specific for a particular differentiation stage. However, in recent years several forms of lineage infidelity have been described (83,84), including the unexpected rearrangements of Ig and TcR genes (85,86).

Early murine studies revealed that rearrangement and transcription of Ig genes can occur in T cells (87-89). Also in human T cell malignancies Ig gene rearrangements may occur (12,15,21,22,24,27,37,39,40,42,44,45,51,56,58,68,72-75,86,90-97), while TcR gene rearrangements may occur in malignancies of B cell lineage (19-22,27,37,40,43,45,49,51,52,54,56,57,68, 73-75,93,94,98-106). Rearranged Ig and TcR genes have even been found in myeloid malignancies (19,107-117).

The explanation of these cross-lineage rearrangements of Ig and TcR genes probably lies in the control mechanisms for DNA recombination. There is evidence that a common recombinase is involved in the rearrangement of Ig and TcR genes (118). The accessibility of the Ig and TcR genes for this recombinase probably depends on the openess of the chromatine configuration around the genes (85). Progenitors of lymphoid cells or more primitive multipotential (myeloid/lymphoid) progenitors might rearrange their Ig or TcR genes before lineage commitment. As soon as an irreversible commitment to T cell, B cell or myeloid lineage has occurred, the progenitor cell adapts its phenotype by down-regulating and up-regulating the expression of differentiation antigens, while the cross-lineage gene rearrangements remain as an "artefact".

Therefore, Ig and TcR gene rearrangements cannot be used as markers for B cell and T cell lineage, respectively. Nevertheless, the combined use of information about morphology, immunophenotype and the configuration of Ig and TcR genes will generally provide sufficient information about the differentiation lineage of a hematopoietic malignancy.

Ig gene rearrangements in T cell malignancies

IgH gene rearrangements occur in 10 to 15% of lymphoblastic T cell malignancies, while in the more mature T cell malignancies such as chronic T cell leukemias and peripheral T cell lymphomas this frequency is about 5% (Table 4). The IgH gene rearrangements in T cell malignancies involve only one allele in the majority of cases (12 out of 14 cases; i.e. 86%) (21,22,27,42,44,56,68,72,92).

IgL gene rearrangements appear to be rare in T cell malignancies. Rearranged Igκ genes

TABLE 4. Ig gene rearrangements in T cell malignancies[a].

	Rearranged IgH genes	Rearranged Igκ genes	Rearranged Igλ genes
Lymphoblastic T cell malignancies			
$CD3^-$ T-ALL	16% (12/76)	0% (0/30)	0% (0/29)
$CD3^+$ T-ALL	6% (3/49)	0% (0/29)	0% (0/28)
all T-ALL together	14% (26/180)	0% (0/62)	0% (0/59)
T-NHL	10% (2/20)	33% (2/6)[b]	0% (0/1)
Mature T cell malignancies			
T-CLL	3% (1/32)	0% (0/4)	0% (0/4)
T-PLL	7% (1/14)		
ATLL	3% (1/30)	0% (0/3)	0% (0/3)
peripheral T-NHL	4% (2/45)	12% (2/17)[b]	0% (0/2)
Lennert lymfoom	0% (0/5)	0% (0/5)	
CTLL/MF	0% (0/14)	0% (0/1)	
SS	7% (1/15)	0% (0/5)	0% (0/5)

a. The data about the frequency of Ig gene rearrangements in T cell malignancies are derived from refs. 12,15,21,22,24,27,37,39,40,42,44, 45,51,56,58,68,72-75,86,90-97. Several research groups have published more than one report on rearranged Ig genes in T cell malignancies. To avoid double counting we have only included the most informative data of each research group: ref. 68, leaving out refs. 15,40,51,90 and 91; ref. 73, leaving out refs. 74 and 75; combination of refs. 93 and 94; ref. 96, leaving out ref. 95. For abbreviations: see Table 3, footnotes.

b. Igκ gene rearrangements were found in only a few T cell malignancies, i.e. two lymphoblastic T-NHL (95-97) and two peripheral T-NHL (97). Remarkably one lymphoblastic T-NHL had rearranged Igκ genes but germline IgH genes (97).

were found in only two lymphoblastic T-NHL and two peripheral T-NHL (95-97). Remarkably, one of the lymphoblastic T-NHL had rearranged Igκ genes, but germline IgH genes (97). Igλ gene rearrangements have not been reported to occur in T cell malignancies (Table 4).

TcR gene rearrangements in malignancies of B cell lineage

TcR gene rearrangements occur in a relatively high frequency in precursor B-ALL, but their frequency is much lower or they are absent in B-ALL and mature B cell malignancies such as chronic B cell leukemias, B-NHL and multiple myeloma (Table 5). Within the group of precursor B-ALL the frequency of TcR-γ gene rearrangements (~55%) is higher than the frequency of TcR-β gene rearrangements (~33%). Rearrangements and/or deletions of the TcR-δ genes occur even more frequently (~80%) and also TcR-α gene rearrangements are regularly found in precursor B-ALL (~61%) (54,57,105,106). This implies that the cross-lineage rearrangements of TcR genes in precursor B-ALL is much more frequent than the cross-lineage rearrangements of IgH genes in T-ALL (~14%) (Table 4). However, the frequency of TcR-β and TcR-γ gene rearrangements in mature B cell malignancies (0 to 7%) is comparable to the frequency of IgH

TABLE 5. TcR gene rearrangements in malignancies of B cell lineage.

	Rearranged TcR-α genes[a]	Rearranged TcR-β genes	Rearranged TcR-γ genes	Rearranged TcR-δ gene	Deleted TcR-δ genes
Lymphoblastic B cell malignancies					
null ALL ($CD10^-$, $Cy\mu^-$)[b]	0% (0/2)	9% (3/33)	22% (7/32)	0% (0/6)	33% (2/6)
common ALL ($CD10^+$, $Cy\mu^-$)[c]		41% (66/162)	66% (88/134)	54% (13/24)	25% (6/24)
pre-B-ALL ($CD10^+$, $Cy\mu^+$)[c]		23% (10/44)	44% (18/41)	20% (2/10)	0% (0/10)
$CD10^+$ precursor B-ALL[b]	63% (17/27)	35% (94/268)	59% (146/246)	52% (32/62)	27% (17/62)
all precursor B-ALL[b]	61% (17/29)	33% (105/318)	55% (160/289)	54% (52/97)	26% (25/97)
B-ALL ($SmIg^+$)[b]		0% (0/7)	0% (0/8)		
Mature B cell malignancies[d]					
B-CLL		7% (11/165)	0% (0/116)	0% (0/15)	0% (0/15)
B-PLL		0% (0/10)	0% (0/8)	0% (0/11)	0% (0/11)
HCL		0% (0/9)	0% (0/9)	0% (0/7)	0% (0/7)
B-NHL		6% (10/158)	6% (6/96)		
multiple myeloma		0% (0/5)			

a. The data about TcR-α gene rearrangements in precursor B-ALL are derived from ref. 105.

b. The data about TcR gene rearrangements in null ALL, $CD10^+$ precursor B-ALL, all precursor B-ALL and B-ALL are derived from refs. 19-22,43,45,49,52,54,57,68,94,95,99,100,104,106,107. Several research groups have published more than once data on TcR gene rearrangements in precursor B-ALL. To avoid double counting, we have selected the most informative data from each research group: the data from refs. 54,68 and 105 were combined; the data from refs. 20 and 21 were combined; data from ref. 94 were used, while data from ref. 93 were excluded.

c. The data about TcR gene rearrangements in common ALL ($CD10^+$, $Cy\mu^-$) and pre-B-ALL ($CD10^+$, $Cy\mu^+$) are derived from refs. 19,40,51,68,103 and 106. From ref. 51 we only used the TcR-γ data.

d. The data about TcR gene rearrangements in mature B cell malignancies are derived from refs. 27,37,45,49,52,56,73-75,93,94,98,100-102. Several research group have published more than one report about TcR gene rearrangements in mature B cell malignancies. To avoid double counting we have selected the most informative information from each research group: data from ref. 75 were used, while data from refs. 73 and 74 were excluded; data from refs. 49 and 56 were combined; only data from ref. 94 were used, while data from ref. 93 were excluded.

gene rearrangements in mature T cell malignancies (0 to 7%) (Tables 4 and 5) (27,37,45,49, 52,56,73-75,93,94,98,100-102).

Assuming that the rate of Ig and TcR gene rearrangements in leukemic cells reflects the rate in normal cells, it is intriguing to find less frequent cross-lineage gene rearrangements in mature malignancies than in immature lymphoid malignancies (Tables 4 and 5). This can be explained by assuming that the common recombinase is still active in ALL cells, especially precursor B-ALL cells, but that the activity of this enzyme is absent in mature lymphoid malignancies (98). Alternatively, it may be that the cross-lineage Ig and TcR gene rearrangements occur frequently in normal T and B cell progenitors (and their malignant counterparts), but that such cells are less able to differentiate and to expand than normal progenitors without cross-lineage gene rearrangements (98). This is in line with data that suggests that only a small part of the lymphoid precursor cells reach a mature stage of differentiation. Also non-functional intra-lineage Ig and TcR gene rearrangements may cause the abortive events.

TABLE 6. Comparison of TcR gene rearrangements in precursor B-ALL and T-ALL.

	precursor B-ALL	T-ALL
Rearranged TcR-β genes		
- monoallelic β1 rearrangement	0% (0/44)[a]	5% (4/80)[b]
- biallelic β2 rearrangement	43% (19/44)[a]	26% (21/80)[b]
Rearranged TcR-γ genes		
- monoallelic γ1 rearrangement	51% (39/76)[c]	3% (1/38)[d]
- biallelic γ2 rearrangement	9% (7/76)[c]	82% (31/38)[d]
Rearranged TcR-γ genes, but germline TcR-β genes	28% (76/272)[e]	rare (<5%)[f]
Rearranged or deleted TcR-δ genes, but germline TcR-β and TcR-γ genes	16% (16/97)[g]	rare (<5%)[f]

a. Data derived from refs. 22,68,98 and 103.
b. Data derived from refs. 21,34,40,41,46,47,49 and 59.
c. Data derived from refs. 22,43,68,98 and 103.
d. Data derived from refs. 43,49,51 and 59.
e. Data derived from refs. 19,21,22,43,45,57,68,98,103 and 106.
f. See Table 3.
g. Data derived from refs. 54,57 and 106.

The frequencies of cross-lineage TcR-β and TcR-γ gene rearrangements appear to be different for the various types of precursor B-ALL. TcR-β and TcR-γ gene rearrangements are infrequent in null ALL ($CD10^-$, $Cy\mu^-$), occur in higher frequencies in pre-B-ALL ($CD10^+$,$Cy\mu^+$), and are found most frequently in common ALL ($CD10^+$,$Cy\mu^-$) (Table 5) (19,40,51,68,103,106).

Several reports indicate that the frequency of TcR gene rearrangements in precursor B-ALL is also influenced by the age of the patient. TcR-β and TcR-γ gene rearrangements occur in low frequencies in precursor B-ALL of young children (<2 y), especially when it concerns $CD10^-$ precursor B-ALL (20,21,104). The frequency of the cross-lineage TcR gene rearrangements is higher in precursor B-ALL of older children (>2 y), while the highest frequencies are reported to occur in adult precursor B-ALL (103,104).

The configurations of rearranged TcR-γ genes in precursor B-ALL are strikingly different from those in T-ALL (Table 6) (22,43,68,98,103). In precursor B-ALL a large part of the TcR-γ gene rearrangements (~51%) involve the γ1 locus on one allele, while biallelic rearrangements to the γ2 locus are infrequent (~9%). This is in contrast to the high frequency of biallelic γ2 gene rearrangements and the low frequency of monoallelic γ1 gene rearrangements in T-ALL (Table 6) (43,49,51,52,59). Such a large discrepancy is not found when the configurations of the rearranged TcR-β genes are compared (22,68,98,103).

In the reported series of T-ALL only a few T-ALL with rearranged TcR-γ genes but germline TcR-β genes occur and also T-ALL with rearranged TcR-δ genes but germline TcR-β and TcR-γ genes are rare (Table 3). However, about 28% of precursor B-ALL have rearranged TcR-γ genes with germline TcR-β genes and about 16% of precursor B-ALL have rearranged or deleted TcR-δ genes with germline TcR-β and TcR-γ genes (Table 6)(19,21,22,43, 45,54,57,68, 98,103,106). If one assumes that also the cross-lineage TcR gene rearrangements occur in a

hierarchic order, these findings support the T cell differentiation model in which TcR-δ genes rearrange first, followed by TcR-γ gene rearrangements (γ1 rearrangements prior to γ2 rearrangements) and subsequently by TcR-β gene rearrangements (Figure 4).

The position of the TcR-α genes in the hierarchic order of TcR gene rearrangements is difficult to determine, because too few data are available concerning TcR-α gene rearrangements in precursor B-ALL.

Most precursor B-ALL with rearranged TcR-α genes (11 out of 17) have rearranged or deleted TcR-δ genes as well as rearranged TcR-γ genes and/or rearranged TcR-β genes. However, some of them have germline TcR-γ and TcR-β genes (105). This supports the hypothesis that rearrangement of TcR-δ genes and deletion of TcR-δ genes (followed by rearrangement of TcR-α genes) are early events in T cell differentiation, which probably play an important role in the bifurcation of TcR-$\gamma\delta$ and TcR-$\alpha\beta$ pathways (see Chapters 3.2 and 4.1).

Ig and TcR gene rearrangements in acute myeloid leukemias

Ig and TcR gene rearrangements also occur in acute myeloid leukemias (AML) (19,107-117). Their frequencies are lower than the cross-lineage rearrangements of TcR gene rearrangements in precursor B-ALL, but comparable to the frequencies of Ig gene rearrange-

TABLE 7. Ig and TcR gene rearrangements in AML.

	Rearranged						
Investigators[a]	IgH genes	Igκ genes	Igλ genes	TcR-β genes	TcR-γ genes	TcR-δ genes	Total
Rovigatti et al.(107)	2/14						2/14
Ha et al.(108)	1/19	0/19	0/19				1/19
Cheng et al.(109)	1/24[b]	0/24		3/24[b]			3/24
Foa et al.(110)	10/52[c]	3/46[c]	0/26	1/47[c,d]	4/51[c,d]		10/52
Boehm et al.(111)	3/17[e]	0/17	0/17	3/17[d]	12/17[d]		13/17
Chen et al.(98)				0/9	0/9		0/9
Serementis et al.(113)	5/38[f]			6/38[f]			10/38
Goorha et al.(19)	2/12			0/12	0/12		2/12
Wainscoat et al.(119)	0/17			0/17	0/17		0/17
Oster et al.(115)	10/69[g]	1/69[g]		3/69[g]			11/69
Fontenay et al.(116)	11/57[h]			4/57[h]	3/57[h]	8/57[h]	13/57
TOTAL	45/319	24/175	0/62	20/290	19/163	8/57	65/328
percentage	14%	2%	0%	7%	12%	14%	20%

a. Only reports which describe at least nine AML were used for this table. All AML were diagnosed according to the FAB criteria and the investigators did not mention selection criteria for inclusion in the study. Therefore we assume that the AML listed in this table are a representative sample.
b. One AML had rearranged IgH and rearranged TcR-β genes (109).
c. All AML with rearranged TcR-β, TcR-γ and/or Igκ genes had also rearranged IgH genes (110).
d. All AML with rearranged TcR-β genes also had rearranged TcR-γ genes (110,111).
e. Two AML with rearranged IgH genes had rearranged TcR-γ genes and one out of them also had rearranged TcR-β genes (111).
f. One AML had rearranged TcR-β genes as well as rearranged IgH genes (113).
g. Two AML with rearranged IgH genes had rearranged TcR-β genes and one of them also had rearranged Igκ genes (115).
h. Six AML with rearranged IgH genes, all four AML with rearranged TcR-β genes and all three AML with rearranged TcR-γ genes also had rearranged TcR-δ genes (116).

TABLE 8. Ig AND TcR GENE REARRANGEMENTS IN TdT^+ AML AND TdT^- AML[a].

	Rearranged IgH genes	Rearranged Igκ genes	Rearranged TcR-β genes	Rearranged TcR-γ genes	Total (rearranged Ig and/or TcR genes)
TdT^+ AML[b]	57% (24/42)	9% (1/11)	23% (9/40)	35% (6/17)	71% (30/42)
TdT^- AML[b]	5% (7/143)	0% (0/58)	1% (2/135)	2% (1/52)	6% (8/143)

a. The data are derived from refs. 107,110,112-115. It should be remarked that in at least two of the four reports the investigators have selected for TdT^+ AML (107,114).
b. An AML was considered to be TdT^+, when the percentage of TdT^+ cells was ≥ 25%.

ments in T-ALL (Table 7) (19,98,107-111,113,115,116,119). Analogous to the latter, also in AML IgH gene rearrangements occur more frequently than IgL gene rearrangements and TcR-γ gene rearrangements occur more frequently than TcR-β gene rearrangements. Furthermore, all AML with rearranged Igκ genes also had rearranged IgH genes and virtually all AML with rearranged TcR-β genes had rearranged TcR-γ genes (110,111,115,116). Like in precursor B-ALL the TcR-γ gene rearrangements in AML mainly represent monoallelic rearrangements to the γ1 locus (110,111). This cross-lineage hierarchic order of gene rearrangements in AML is in line with the normal hierarchic order of Ig and TcR gene rearrangements during lymphoid differentiation (Figure 4).

Several reports suggest that Ig and TcR gene rearrangements occur more frequently in TdT^+ AML than in TdT^- AML (Table 8) (107,110,112-115). Although TdT probably plays an important role during Ig and TcR gene rearrangements by inserting nucleotides at junction sites (120-122), this enzyme has not been shown to be capable of initiating or mediating the process of gene rearrangement. Therefore it is not surprising that several recent studies on large series of AML do not find a significant relationship between TdT expression and Ig or TcR gene rearrangements in AML (116,117; H.J. Adriaansen, unpublished results).

It has also been suggested that cross-lineage gene rearrangements are rare in adult AML of FAB type M4 and M5 (123). This is in contrast with other reports, which indicate that Ig and TcR gene rearrangements can occur in any FAB group of AML (107-113,115).

Ig and TcR gene rearrangements in acute undifferentiated leukemias and acute leukemias with ambiguous phenotype

The frequent occurrence of cross-lineage Ig and TcR gene rearrangements in ALL and AML (Table 4-8) hampers their use as markers for lineage in case of acute undifferentiated leukemias (AUL) and acute leukemias with ambiguous phenotype. Several reports describe that a large part of such leukemias have rearranged their IgH genes and that some of them have rearranged IgL genes or rearranged TcR-β genes (16,113,124,125). This illustrates that the combined morphological, immunologic and molecular analyses do not always allow determination of lineage, although in the great majority of leukemias and NHL such analysis provide sufficient information for lineage assignment.

CONCLUSION

The great majority of lymphoid malignancies of B and T cell origin have clonally rearranged Ig and TcR genes, respectively. Analysis of these malignancies has given more insight in the hierarchic order Ig and TcR gene rearrangements during lymphoid differentiation.

Also cross-lineage Ig and TcR gene rearrangements occur. This especially concerns TcR gene rearrangements in precursor B-ALL (30 to 80%, depending on the type of TcR gene), IgH gene rearrangements in T-ALL (5 to 15%) and Ig and/or TcR gene rearrangements in AML (~20% of all AML).

Both the appropriate and cross-lineage Ig and TcR gene rearrangements in hematopoietic malignancies can be used for diagnostic purposes (see Chapter 6.3).

ACKNOWLEDGMENTS. We gratefully acknowledge Professor Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Mr. T.M. van Os (Department of Immunology, Erasmus University, Rotterdam) and Mr. C. van Dijk (Interpoint, Hapert) for excellent assistance in the preparation of the figures and Mrs. J. de Goey-van Dooren, Ms. E.P. Schenkel and Ms. H.J. de Jager for their secretarial assistance.

REFERENCES

1. Janossy G, Bollum FJ, Bradstock KF, Ashley J. Cellular phenotypes of normal and leukemic hemopoietic cells determined by analysis with selected antibody combinations. Blood 1980;56:430-41.
2. Tubbs RR, Fishleder A, Weiss RA, Savage RA, Sebek BA, Weick JK. Immunohistologic cellular phenotypes of lymphoproliferative disorders: comprehensive evaluation of 564 cases including 257 non-Hodgkin's lymphomas classified by the international working formulation. Am J Pathol 1983;113:207-21.
3. Foon KA, Todd III RF. Immunologic classification of leukemia and lymphoma. Blood 1986;68:1-31.
4. Greaves MF. Differentiation-linked leukemogenesis in lymphocytes. Science 1986;234:697-704.
5. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.
6. Mason DY. A new look at lymphoma immunohistology. Am J Pathol 1987;128:1-4.
7. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987;128:181-201.
8. Schuurman H-J, Van Baarlen J, Huppes W, Lam BW, Verdonck LF, Van Unnik JAM. Immunophenotyping of non-Hodgkin's lymphoma: lack of correlation between immuno-phenotype and cell morphology. Am J Pathol 1987;129:140-51.
9. Picker LJ, Brenner MB, Weiss LM, Smith SD, Warnke RA. Discordant expression of CD3 and T-cell receptor beta-chain antigens in T-lineage lymphomas. Am J Pathol 1987;129:434-40.
10. Weiss LM, Crabtree GS, Rousse RV, Warnke RA. Morphologic and immunologic characterization of 50 peripheral T-cell lymphomas. Am J Pathol 1985;118:316-24.
11. Drexler HG. Classification of acute myeloid leukemias - a comparison of FAB and immunophenotyping. Leukemia 1987;1:697-705.
12. Korsmeyer SJ, Arnold A, Bakhshi A, Ravetch JV, Siebenlist U, Hieter PA, Sharrow SO, LeBien TW, Kersey JH, Poplack DG, Leder P, Waldmann TA. Immunoglobulin gene rearrangement and cell surface antigen expression in acute lymphocytic leukemias of T cell and B cell precursor origins. J Clin Invest 1983;71:301-13.
13. Nadler LM, Korsmeyer SJ, Anderson KC, Boyd AW, Slaughenhoupt B, Park E, Jensen J, Coral F, Mayer RJ, Sallan SE, Ritz J, Schlossman SF. B cell origin of non-T cell acute lymphoblastic leukemia: a model for discrete stages of neoplastic and normal pre-B cell differentiation. J Clin Invest 1984;74:332-40.
14. Foa R, Migone N, Saitta M, Fierro MT, Giubellino MC, Lusso P, Cordero di Montezemolo L, Miniero R, Lauria

F. Different stages of B cell differentiation in non-T acute lymphoblastic leukemia. J Clin Invest 1984;74:1756-63.
15. Ha K, Hozumi N, Hrincu A, Gelfand EW. Lineage specific classification of leukaemia: results of the analysis of sixty cases of childhood leukaemia. Br J Haematol 1985;61:237-49.
16. Raghavachar A, Bartram CR, Ganser A, Heil G, Kleihauer E, Kubanek B. Acute undifferentiated leukemia: implications for cellular origin and clonality suggested by analysis of surface markers and immunoglobulin gene rearrangement. Blood 1986;68:658-62.
17. Foa R, Migone N, Basso G, Cattoretti G, Pizzolo G, Lauria F, Casorati G, Giubellino MC, Capuzzo F, Cantù-Rajnoldi A, Lusso P, Carbonara AO, Gavosto F. Molecular and immunological evidence of B-cell commitment in "null" acute lymphoblastic leukaemia. Int J Cancer 1986;38:317-23.
18. Foa R, Migone N, Fierro MT, Basso G, Lusso P, Putti MC, Giubellino MC, Saitta M, Miniero R, Casorati G, Gavosto F. Genotypic characterization of common acute lymphoblastic leukemia may improve the phenotypic classification. Exp Hematol 1987;15:942-5.
19. Goorha R, Bunin N, Mirro J Jr, Murphy SB, Cross AH, Behm FG, Quertermous T, Seidman J, Kitchingman GR. Provocative pattern of rearrangements of the genes for the γ and β chains of the T-cell receptor in human leukemias. Proc Natl Acad Sci USA 1987;84:4547-51.
20. Felix CA, Reaman GH, Korsmeyer SJ, Hollis GF, Dinndorf PA, Wright JJ, Kirsch IR. Immunoglobulin and T cell receptor gene configuration in acute lymphoblastic leukemia of infancy. Blood 1987;70:536-41.
21. Felix CA, Wright JJ, Poplack DG, Reaman GH, Cole D, Goldman P, Korsmeyer SJ. T cell receptor α-, β-, and γ-genes in T cell and pre-B cell acute lymphoblastic leukemia. J Clin Invest 1987;80:545-56.
22. Boehm TLJ, Werle A, Ganser A, Kornhuber B, Drahovsky D. T cell receptor γ chain variable gene rearrangements in acute lymphoblastic leukemias of T and B lineage. Eur J Immunol 1987;17:1593-7.
23. Korsmeyer SJ, Greene WC, Cossman J, Hsu S-M, Jensen JP, Neckers LM, Marshall SL, Bakhshi A, Depper JM, Leonard WJ, Jaffe ES, Waldmann TA. Rearrangement and expression of immunoglobulin genes and expression of Tac antigen in hairy cell leukemia. Proc Natl Acad Sci USA 1983;80:4522-6.
24. Foroni L, Catovsky D, Rabbitts TH, Luzzatto L. DNA rearrangements of immunoglobulin genes correlate with phenotypic markers in B-cell malignancies. Mol Biol Med 1984;2:63-79.
25. Cleary ML, Wood GS, Warnke R, Chao J, Sklar J. Immunoglobulin gene rearrangements in hairy cell leukemia. Blood 1984;64:99-104.
26. Foroni L, Catovsky D, Luzzatto L. Immunoglobulin gene rearrangements in hairy cell leukemia and other chronic B cell lymphoproliferative disorders. Leukemia 1987;1:389-92.
27. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE Jr, Whitcomb CC, Thomas IV CY. Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 1987;69:79-86.
28. Aisenberg AC, Wilkes BM, Jacobson JO, Harris NL. Immunoglobulin gene rearrangements in adult non-Hodgkin's lymphoma. Am J Med 1987;82:738-44.
29. Korsmeyer SJ, Hieter PA, Sharrow SO, Goldman CK, Leder P, Waldmann TA. Normal human B cells display ordered light chain gene rearrangements and deletions. J Exp Med 1982;156:975-85.
30. Alt FW, Yancopoulos GD, Blackwell TK, Wood C, Thomas E, Boss M, Coffman R, Rosenberg N, Tonegawa S, Baltimore D. Ordered rearrangement of immunoglobulin heavy chain variable region segments. EMBO J 1984;3:1209-19.
31. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus, in press.
32. Ohashi PS, Mak TW, Van den Elsen P, Yanagi Y, Yoshikai Y, Calman AF, Terhorst C, Stobo JD, Weiss A. Reconstitution of an active surface T3/T-cell antigen receptor by DNA transfer. Nature 1985;316:606-9.
33. Van Dongen JJM, Geurts van Kessel AHM, Wolvers-Tettero ILM, Versnel MA, Van Oudenaren A, Schoenmaker E, Hagemeijer A. Construction of human-mouse T cell hybrids that express human T cell-associated surface antigens and allow the chromosomal localization of these antigens. J Immunol 1986;137:1047-53.
34. Royer HD, Acuto O, Fabbi M, Tizard R, Ramachandran K, Smart JE, Reinherz EL. Genes encoding the Ti β subunit of the antigen/MHC receptor undergo rearrangement during intrathymic ontogeny prior to surface T3-Ti expression. Cell 1984;39:261-6.
35. Minden MD, Toyonaga B, Ha K, Yanagi Y, Chin B, Gelfand E, Mak T. Somatic rearrangement of T-cell antigen

receptor gene in human T-cell malignancies. Proc Natl Acad Sci USA 1985;82:1224-7.
36. Flug F, Pelicci P-G, Bonetti F, Knowles II DM, Dalla-Favera R. T-cell receptor gene rearrangements as markers of lineage and clonality in T-cell neoplasms. Proc Natl Acad Sci USA 1985;82:3460-4.
37. O'Connor NTJ, Weatherall DJ, Feller AC, Jones D, Pallesen G, Stein H, Wainscoat JS, Gatter KC, Isaacson P, Lennert K, Ramsey A, Wright DH, Mason DY. Rearrangement of the T-cell-receptor β-chain gene in the diagnosis of lymphoproliferative disorders. Lancet 1985;i:1295-7.
38. Rabbitts TH, Stinson A, Forster A, Foroni L, Luzzato L, Catovsky D, Hammerström L, Smith CIE, Jones D, Karpas A, Minowada J, Taylor AMR. Heterogeneity of T-cell β-chain gene rearrangements in human leukemias and lymphomas. EMBO J 1985;4:2217-24.
39. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 1985;313:776-83.
40. Tawa A, Hozumi N, Minden M, Mak TW, Gelfand EW. Rearrangement of the T-cell receptor β-chain gene in non-T-cell, non-B-cell acute lymphoblastic leukemia of childhood. N Engl J Med 1985;313:1033-7.
41. Furley AJ, Mizutani S, Weilbaecher K, Dhaliwal HS, Ford AM, Chan LC, Molgaard HV, Toyonaga B, Mak T, Van den Elsen P, Gold D, Terhorst C, Greaves MF. Developmentally regulated rearrangement and expression of genes encoding the T cell receptor-T3 complex. Cell 1986;46:75-87.
42. Pittaluga S, Raffeld M, Lipford EH, Cossman J. 3A1 (CD7) expression precedes Tβ gene rearrangements in precursor T (lymphoblastic) neoplasms. Blood 1986;68:134-9.
43. Greenberg JM, Quertermous T, Seidman JG, Kersey JH. Human T cell γ-chain gene rearrangements in acute lymphoid and nonlymphoid leukemia: comparison with the T cell receptor β-chain gene. J Immunol 1986;137:2043-9.
44. Suzuki H, Ueda R, Obata Y, Seto M, Hirata R, Iwamura H, Ota K, Takahashi T. T cell receptor β chain gene rearrangements in leukemias with immature T cell phenotype. Jpn J Cancer Res 1986;77:1069-73.
45. Davey MP, Bongiovanni KF, Kaulfersch W, Quertermous T, Seidman JG, Hershfield MS, Kurtzberg J, Haynes BF, Davis MM, Waldmann TA. Immunoglobulin and T-cell receptor gene rearrangement and expression in human lymphoid leukemia cells at different stages of maturation. Proc Natl Acad Sci USA 1986;83:8759-63.
46. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. T cell receptor-CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA, and cell membrane level. J Immunol 1987;138:1260-9.
47. Mirro J Jr, Kitchingman G, Behm FG, Murphy SB, Goorha RM. T cell differentiation stages identified by molecular and immunologic analysis of the T cell receptor complex in childhood lymphoblastic leukemia. Blood 1987;69:908-12.
48. Miwa H, Konishi H, Kobayashi N, Kita K, Shirakawa S, Shimizu A, Honjo T, Hatanaka M. The T-cell receptor gene rearrangements in T-lineage tumors without OKT-3,4,6,8 markers. J Mol Cell Immunol 1987;3:37-42.
49. Foroni L, Foldi J, Matutes E, Catovsky D, O'Connor NJ, Baer R, Forster A, Rabbitts TH, Luzzatto L. α, β and γ T-cell receptor genes: rearrangements correlate with haematological phenotype in T cell leukaemias. Br J Haematol 1987;67:307-18.
50. Le Paslier D, Chen Z, Loiseau P, Cohen D, Sigaux F. T cell rearranging gene γ: diversity and mRNA expression in fresh cells from T cell acute lymphoblastic leukemia. Blood 1987;70:637-46.
51. Tawa A, Benedict SH, Hara J, Hozumi N, Gelfand EW. Rearrangement of the T cell receptor γ-chain gene in childhood acute lymphoblastic leukemia. Blood 1987;70:1933-9.
52. Subar M, Pelicci PG, Neri A, Allavena P, Littman DR, Knowles II DM, Dalla-Favera R. Patterns of T cell receptor gamma gene rearrangement and expression in B and T lymphoid malignancies. Leukemia 1988;2:19-26.
53. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Comparison of T cell receptor α, β, and γ gene rearrangement and expression in T cell acute lymphoblastic leukemia. J Clin Invest 1988;81:989-96.
54. Hara J, Benedict SH, Champagne E, Takihara Y, Mak TW, Minden M, Gelfand EW. T cell receptor δ gene rearrangements in acute lymphoblastic leukemia. J Clin Invest 1988;82:1974-82.
55. Hockett RD, De Villartay J-P, Pollock K, Poplack DG, Cohen DI, Korsmeyer SJ. Human T-cell antigen receptor

(TCR) δ-chain locus and elements responsible for its deletion are within the TCR α-chain locus. Proc Natl Acad Sci USA 1988;85:9694-8.
56. Foroni L, Laffan M, Boehm T, Rabbitts TH, Catovsky D, Luzzatto L. Rearrangement of the T-cell receptor δ genes in human T-cell leukemias. Blood 1989;73:559-65.
57. Loiseau P, Guglielmi P, Le Paslier D, Macintyre E, Gessain A, Bories J-C, Flandrin G, Chen Z, Sigaux F. Rearrangements of the T cell receptor δ gene in T acute lymphoblastic leukemia cells are distinct from those occurring in B lineage acute lymphoblastic leukemia and preferentially involve one Vδ gene segment. J Immunol 1989;142:3305-11.
58. Biondi A, Champagne E, Rossi V, Giudici G, Cantù-Rajnoldi A, Masera G, Mantovani A, Mak TW, Minden MD. T-cell receptor delta gene rearrangement in childhood T-cell acute lymphoblastic leukemia. Blood 1989;73:2133-8.
59. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T-cell receptor delta genes in T-cell acute lymphoblastic leukemias. Blood 1989;74:334-42.
60. De Vilartay J-P, Pullman AB, Andrade R, Tschachler E, Colamenici O, Neckers L, Cohen DI, Cossman J. γ/δ Lineage relationship within a consecutive series of human precursor T-cell neoplasms. Blood 1989;74:2508-18.
61. Baer R, Boehm T, Yssel H, Spits H, Rabbitts TH. Complex rearrangements within the human Jδ-Cδ/Jα-Cα locus and aberrant recombination between Jα segments. EMBO J 1988;7:1661-8.
62. Champagne E, Sagman U, Biondi A, Lewis WH, Mak TW, Minden MD. Structure and rearrangement of the T cell receptor J alpha locus in T cells and leukemic T cell lines. Eur J Immunol 1988;18:1033-8.
63. Hayday AC, Diamond DJ, Tanigawa G, Heilig JS, Folsom V, Saito H, Tonegawa S. Unusual organization and diversity of T-cell receptor α-chain genes. Nature 1985;316:828-32.
64. Yoshikai Y, Clark SP, Taylor S, Sohn U, Wilson BI, Minden MD, Mak TW. Organization and sequences of the variable, joining and constant region genes of the human T-cell receptor α-chain. Nature 1985;316:837-40.
65. Griesser H, Champagne E, Tkachuk D, Takihara Y, Lalande M, Baillie E, Minden M, Mak TW. The human T cell receptor α-δ locus: a physical map of the variable, joining and constant region genes. Eur J Immunol 1988;18:641-4.
66. Baer R, Lefranc M-P, Minowada J, Foster A, Stinson MA, Rabbitts TH. Organization of the T-cell receptor α-chain gene and rearrangement in human T-cell leukaemias. Mol Biol Med 1986;3:265-77.
67. Sangster RN, Minowada J, Suciu-Foca N, Minden M, Mak TW. Rearrangement and expression of the α, β, and γ chain T cell receptor genes in human thymic leukemia cells and functional T cells. J Exp Med 1986;163:1491-508.
68. Hara J, Benedict SH, Mak TW, Gelfand EW. T cell receptor α-chain gene rearrangements in B-precursor leukemia are in contrast to the findings in T cell acute lymphoblastic leukemia: comparative study of T cell receptor gene rearrangement in childhood leukemia. J Clin Invest 1987;80:1770-7.
69. Bertness V, Kirsch I, Hollis G, Johnson B, Bunn PA. T-cell receptor gene rearrangements as clinical markers of human T-cell lymphomas. N Engl J Med 1985;313:534-8.
70. Weiss LM, Hu E, Wood GS, Moulds G, Cleary ML, Warnke R, Sklar J. Clonal rearrangements of T-cell receptor genes in mycosis fungoides and dermatopathic lymphadenopathy. N Engl J Med 1985;313:539-44.
71. Foa R, Pelicci P-G, Migone N, Laura F, Pizzolo G, Flug F, Knowles II DM, Dalla-Favera R. Analysis of T-cell receptor beta chain (Tβ) gene rearrangements demonstrates the monoclonal nature of T-cell chronic lymphoproliferative disorders. Blood 1986;67:247-50.
72. Matsuoka M, Hagiya M, Hattori T, Asou N, Mueda S, Shimada K, Tsai SC, Sakano H, Takatsuki K. Gene rearrangements of T cell receptor β and γ chains in HTLV-I infected primary neoplastic T cells. Leukemia 1988;2:84-90.
73. Griesser H, Feller A, Lennert K, Minden M, Mak TW. Rearrangement of the β chain of the T cell antigen receptor and immunoglobulin genes in lymphoproliferative disorders. J Clin Invest 1986;78:1179-84.
74. Griesser H, Feller A, Lennert K, Tweedale M, Messner HA, Zalcberg J, Minden MD, Mak TW. The structure of the T cell gamma chain gene in lymphoproliferative disorders and lymphoma cell lines. Blood 1986; 68:592-4.
75. Tkachuk DC, Griesser H, Takihara Y, Champagne E, Minden M, Feller AC, Lennert K, Mak TW. Rearrangement of T-cell δ locus in lymphoproliferative disorders. Blood 1988;72:353-7.

76. Thiel E, Kranz BR, Raghavachar A, Bartram CR, Löffler H, Messerer D, Ganser A, Ludwig W-D, Büchner T, Hoelzer D. Prethymic phenotype and genotype of pre-T ($CD7^+/ER^-$)-cell leukemia and its clinical significance within adult acute lymphoblastic leukemia. Blood 1989;73:1247-58.
77. Raghavachar A, Thiel E, Hansen-Hagge TE, Kranz B, Bartram C-R. Rearrangement of T cell receptor β, γ, and δ gene loci in human pre-T cell acute lymphoblastic leukemia. Leukemia 1989;3:413-8.
78. Matsuoka M, Asou N, Hattori T, Matsuo T, Nishimura H, Takatsuki K. T cell γ chain gene rearrangement without T cell receptor β chain gene rearrangement in two cases of non-Hodgkin's lymphoma. Acta Haematol 1987;77:172-6.
79. Weiss LM, Picker LJ, Grogan TM, Warnke RA, Sklar J. Absence of clonal beta and gamma T-cell receptor gene rearrangements in a subset of peripheral T-cell lymphomas. Am J Pathol 1988;130:436-42.
80. Ogawa H, Sugiyama H, Oka Y, Maeda T, Komori T, Tani Y, Miyake S, Soma T, Maekura R, Yutani C, Masaoka T, Kishimoto S. Rearrangement of immunoglobulin heavy chain genes and T3 expression in the absence of rearrangement of T-cell receptor β-chain gene in a patient with T-cell malignant lymphoma. Leuk Res 1986;12:1369-75.
81. González-Sarmiento R, LeBien TW, Bradley JG, Greenberg JM, Seidman JG, Ang S, Kersey JH. Acute leukemia expressing the gamma gene product of the putative second T cell receptor. J Clin Invest 1987;79:1281-4.
82. Foroni L, Matutes E, Foldi J, Morilla R, Rabbitts Th, Luzzatto L, Catovsky D. T-cell leukemias with rearrangement of the γ but not β T-cell receptor genes. Blood 1988:71:356-62.
83. Smith LJ, Curtis JE, Messner HA, Senn JS, Furthmayr H, McCulloch EA. Lineage infidelity in acute leukemia. Blood 1983;61:1138-45.
84. Greaves MF, Chan LC, Furley AJW, Watt SM, Molgaard HV. Lineage promiscuity in hemopoietic differentiation and leukemia. Blood 1986;67:1-11.
85. Greaves MF, Furley AJW, Chan LC, Ford AM, Molgaard HV. Inappropriate rearrangement of immunoglobulin and T-cell receptor genes. Immunol Today 1987;8:115-6.
86. Furley AJW, Chan LC, Mizutani S, Ford AM, Weilbaecher K, Pegram SM, Greaves MF. Lineage specificity of rearrangement and expression of genes encoding the T cell receptor-T3 complex and immunoglobulin heavy chain in leukemia. Leukemia 1987;1:644-52.
87. Kemp DJ, Wilson A, Harris AW, Shortman K. The immunoglobulin μ constant region gene is expressed in mouse thymocytes. Nature 1980;286:168-70.
88. Forster A, Hobart M, Hengartner H, Rabbitts TH. An immunoglobulin heavy-chain gene is altered in two T-cell clones. Nature 1980;286:897-9.
89. Kurosawa Y, Von Boehmer H, Haas W, Sakano H, Trauneker A, Tonegawa S. Identification of D segments of immunoglobulin heavy-chain genes and their rearrangement in T lymphocytes. Nature 1981;290:565-70.
90. Ha K, Minden M, Hozumi N, Gelfand EW. Immunoglobulin μ-chain gene rearrangement in a patient with T cell acute lymphoblastic leukemia. J Clin Invest 1984;73:1232-6.
91. Ha K, Minden M, Hozumi N, Gelfand EW. Single allelic Cμ gene rearrangements in patients with T cell and undifferentiated leukemia. Leuk Res 1986;10:1-8.
92. Kitchingman GR, Rovigatti U, Mauer AM, Melvin S, Murphy SB, Stass S. Rearrangement of immunoglobulin heavy chain genes in T cell acute lymphoblastic leukemia. Blood 1985;65:725-9.
93. Pelicci P-G, Knowles II DM, Dalla Favera R. Lymphoid tumors displaying rearrangements of both immunoglobulin and T cell receptor genes. J Exp Med 1985;162:1015-24.
94. Knowles II DM, Neri A, Pelicci PG, Burke JS, Wu A, Winberg CD, Sheibani K, Dalla-Favera R. Immunoglobulin and T-cell receptor β-chain gene rearrangement analysis of Hodgkin's disease: implications for lineage determination and differential diagnosis. Proc Natl Acad Sci USA 1986;83:7942-6.
95. Ha-Kawa K, Hara J, Keiko Y, Muraguchi A, Kawamura N, Ishihara S, Doi S, Yabuuchi H. Kappa-chain gene rearrangement in an apparent T-lineage lymphoma. J Clin Invest 1986;78:1439-42.
96. Ha-Kawa K, Yumura K, Hara J, Ishihara S, Yabuuchi H. Concomitant rearrangements of T-cell β- and γ-chain genes in childhood T-lineage leukemia/lymphoma. Leuk Res 1987;11:739-45.
97. Sheibani K, Wu A, Ben-Ezra J, Stroup T, Rappaport H, Winberg C. Rearrangement of κ-chain and T-cell receptor β-chain genes in malignant lymphomas of "T-cell" phenotype. Am J Pathol 1987;129:201-7.
98. Chen Z, Le Paslier D, Dausset J, Degos L, Flandrin G, Cohen D, Sigaux F. Human T cell γ genes are

frequently rearranged in B-lineage acute lymphoblastic leukemias but not in chronic B cell proliferations. J Exp Med 1987;165:1000-15.

99. Okabe M, Matsushima S, Fukuhara T, Tanaka M, Sakurada K, Kakinuma M, Maekawa I, Miyazaki T. Non-T, non-B acute lymphocytic leukemias: cellular origin based on molecular analyses of immunoglobulin and T-cell α- and β-chain receptor gene rearrangements. Tohoku J Exp Med 1987;152:197-207.
100. Narni F, Colò A, Venturelli D, Selleri L, Donelli A, Tabilio A, Artusi T, Di Prisco U, Torelli G, Torelli U. Immunoglobulin and T-cell receptor beta chain gene rearrangements as lineage markers in human leukemias: a study of 78 cases. Hematologica 1987;72:391-7.
101. Aisenberg AC, Wilkes BM, Jacobson JO. Rearrangement of the genes for the beta and gamma chains of the T cell receptor is rarely observed in adult B cell lymphoma and chronic lymphocytic leukemia. J Clin Invest 1987;80:1209-14.
102. Norton JD, Pattinson J, Hoffbrand AV, Jani H, Yaxley JC, Leber BF. Rearrangement and expression of T cell antigen receptor genes in B cell chronic lymphocytic leukemia. Blood 1988;71:178-85.
103. Norton JD, Campana D, Hoffbrand AV, Janossy G, Coustin-Smith E, Jani H, Yaxley JC, Prentice HG. Correlation of immunophenotype with rearrangement of T cell antigen receptor beta and gamma genes in acute lymphoblastic leukemia of adults. Leukemia 1988;2:27-34.
104. Nuss R, Kitchingman G, Cross A, Zipf TF, Antoun GR, Bernstein I, Behm F, Pullen DJ, Crist W, Mirro J Jr, Goorha R. T cell receptor gene rearrangements in B-precursor acute lymphoblastic leukemia correlate with age and the stage of B cell differentiation. Leukemia 1988;2:722-7.
105. Hara J, Benedict SH, Champagne E, Mak TW, Minden M, Gelfand EW. Relationship between rearrangement and transcription of the T-cell receptor α, β, and γ genes in B-precursor acute lymphoblastic leukemia. Blood 1989;73:500-8.
106. Asou N, Hattori T, Matsuoka M, Kawano F, Takatsuki K. Rearrangements of T-cell antigen receptor δ chain gene in hematologic neoplasms. Blood 1989;74:2707-12.
107. Rovigatti U, Mirro J, Kitchingman G, Dahl G, Ochs J, Murphy S, Stass S. Heavy chain immunoglobulin gene rearrangement in acute nonlymphocytic leukemia. Blood 1984;63:1023-27.
108. Ha K, Minden M, Hozumi N, Gelfand EW. Immunoglobulin gene rearrangement in acute myelogenous leukemia. Cancer Res 1984;44:4658-60.
109. Cheng GY, Minden MD, Toyonaga B, Mak TW, McCulloch EA. T cell receptor and immunoglobulin gene rearrangements in acute myeloblastic leukemia. J Exp Med 1986;163:414-24.
110. Foa R, Casorati G, Giubellino MC, Basso G, Schirò T, Pizzolo G, Lauria F, LeFranc M-P, Rabbitts TH, Migone N. Rearrangements of immunoglobulin and T cell receptor β and γ genes are associated with terminal deoxynucleotidyl transferase expression in acute myeloid leukemia. J Exp Med 1987;165:879-90.
111. Boehm TLJ, Werle A, Drahovsky D. Immunoglobulin heavy chain and T-cell receptor γ and β chain gene rearrangements in acute myeloid leukemias. Mol Biol Med 1987;4:51-62.
112. Lee M-S, Chang K-S, Trujillo JM, McCredie KB, Keating MJ, Freireich EJ, Stass SA. T-cell receptor gamma chain gene rearrangement in acute myelogenous leukemia - evidence for lymphoid lineage prematurity. Hematol Pathol 1987;1:93-8.
113. Seremetis SV, Pelicci P-G, Tabilio A, Ubriaco A, Grignani F, Cuttner J, Winchester RJ, Knowles II DM, Dalla-Favera R. High frequency of clonal immunoglobulin or T cell receptor gene rearrangements in acute myelogenous leukemia expressing terminal deoxyribonucleotidyl transferase. J Exp Med 1987;165:1703-12.
114. Norton JD, Campana D, Hoffbrand AV, Janossy G, Couston-Smith E, Jani H, Yaxley JC, Prentice HG. Rearrangement of immunoglobulin and T cell antigen receptor genes in acute myeloid leukemia with lymphoid-associated markers. Leukemia 1987;1:757-61.
115. Oster W, König K, Ludwig W-D, Ganser A, Lindemann A, Mertelsmann R, Herrmann F. Incidence of lineage promiscuity in acute myeloblastic leukemia: diagnostic implications of immunoglobulin and T-cell receptor gene rearrangement analysis and immunological phenotyping. Leuk Res 1988;12:887-95.
116. Fontenay M, Flandrin G, Baurman H, Loiseau P, Valensi F, Daniel M-T, Sigaux F. T cell receptor δ gene rearrangements occur predominantly in immature myeloid leukemias exhibiting lineage promiscuity. Leukemia 1990;4:100-5.
117. Willière G, Stockinger H, Köller U, Majdic O, Schnabl E, Lutz D, Bettelheim P, Knapp W. Terminal deoxynucleotidyl transferase and CD7 expression in acute myeloid leukemias are not associated with a high

frequency of immunoglobulin and/or T cell receptor gene rearrangement. Leukemia 1990;4:278-81.

118. Yancopoulos GD, Blackwell TK, Suh H, Hood L, Alt FW. Introduced T cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. Cell 1986;44:251-9.
119. Wainscoat JS, Fey MF, Pilkington S, Summers C, Oscier DG. Absence of immunoglobulin and T-cell receptor gene rearrangements in myelodysplastic syndromes and acute nonlymphocytic leukemias. Am J Hematol 1988;28:95-7.
120. Alt FW, Baltimore D. Joining of immunoglobulin heavy chain gene segments: implications from a chromosome with evidence of three D-JH fusions. Proc Natl Acad Sci USA 1982;79:4118-22.
121. Desiderio SV, Yancopoulos GD, Paskind M, Thomas E, Boss MA, Landau N, Alt FW, Baltimore D. Insertion of N regions into heavy-chain genes is correlated with expression of terminal deoxytransferase in B cells. Nature 1984;311:752-5.
122. Landau NR, Schatz DG, Rosa M, Baltimore D. Increased frequency of N-region insertion in a murine pre-B-cell line infected with a terminal deoxynucleotidyl transferase retroviral expression vector. Mol Cell Biol 1987;7:3237-43.
123. Ackland SP, Westbrook CA, Diaz MO, Le Beau MM, Rowley JD. Evidence favoring lineage fidelity in acute nonlymphocytic leukemia: absence of immunoglobulin gene rearrangements in FAB types M4 and M5. Blood 1987;69:87-91.
124. Hara J, Kawa-Ha K, Yumura K, Ishihara S, Doi S, Yabuuchi H, Konishi S, Nishikawa A. Heterogeneity of acute undifferentiated leukemia at the immunoglobulin and T-cell receptor genes level. Jpn J Cancer Res 1987;78:170-5.
125. Ludwig W-D, Bartram CR, Ritter J, Raghavachar A, Hiddemann W, Heil G, Harbott J, Seibt-Jung H, Teichmann JV, Riehm H. Ambiguous phenotypes and genotypes in 16 children with acute leukemia as characterized by multiparameter analysis. Blood 1988;71:1518-28.

CHAPTER 6.3

ANALYSIS OF IMMUNOGLOBULIN AND T CELL RECEPTOR GENES FOR DIAGNOSTIC PURPOSES: POSSIBILITIES AND LIMITATIONS*

J.J.M. van Dongen and I.L.M. Wolvers-Tettero

Department of Immunology, Erasmus University/ University Hospital Dijkzigt, Rotterdam, The Netherlands.

INTRODUCTION

As indicated in Chapter 3.3, Southern blot analysis of immunoglobulin (Ig) and T cell receptor (TcR) genes represents a powerful tool for the detection of clonal rearrangements of these genes. Since malignancies are clonal proliferations, Ig and TcR gene analysis has several diagnostic applications: discrimination between polyclonal and monoclonal lymphoproliferations; detection of two or more subclones within a malignancy at diagnosis; detection of clonal evolution at relapse; analysis of the clonal origin of two lymphoid malignancies in a single patient; and analysis of the differentiation lineage of a malignancy (Table 1). These applications may be extremely useful in the diagnosis and follow-up of lymphoproliferative diseases, but it should be noticed that a clonal cell population does not always represent a malignancy. In this chapter we shall discuss the mentioned diagnostic applications of Ig and TcR gene analysis and pay special attention to the pitfalls and limitations of such analyses.

DISCRIMINATION BETWEEN POLYCLONAL AND MONOCLONAL LYMPHOPROLIFERATIVE DISEASES

Immunologic markers for clonality (e.g. κ/λ ratio) and other cytologic markers are not always sufficiently informative in the evaluation of blood, tissue biopsies or other cell samples for malignancy. This particularly concerns B cell malignancies without Ig light (IgL) chain expression, mature terminal deoxynucleotidyl transferase (TdT) negative T cell malignancies and cell samples in which a small number of malignant lymphoid cells is admixed with large numbers of non-neoplastic lymphoid cells. Analysis of Ig and TcR genes then serves as a sensitive and specific procedure for identifying even minor populations (1 to 5%) of clonal B or T cells.

---

*This manuscript will be published as part of a review in Clin Chim Acta.

TABLE 1. Diagnostic applications of Ig and TcR gene analysis.

1. Discrimination between polyclonal and monoclonal lymphoproliferative diseases.
   CAUTION: monoclonality does not necessarily imply malignancy (e.g. T-γ lymphocytosis and monoclonal or oligoclonal lymphoproliferations in immunodeficiency).
2. Detection of two or more subclones within one malignancy.
3. Analysis of lymphoid malignancies at diagnosis and subsequent relapses:
   - detection of identical Ig and TcR gene rearrangements
   - detection of differences: clonal evolution at first relapse and/or subsequent relapses.
4. Proof or exclusion of the common clonal origin of two malignant lymphoid cell populations.
   CAUTION: one should try to discriminate between two independent lymphoid malignancies and subclone formation within a malignancy.
5. Assignment or exclusion of the differentiation lineage of a malignancy.
6. Detection of low numbers of malignant cells.

B cell non-Hodgkin lymphoma (B-NHL) without IgL chain expression and/or loss of B cell antigens

Like their putative normal counterparts, the majority of B-NHL cells express pan-B cell markers like CD19, CD20, CD22 and CD37 as well as surface membrane Ig (SmIg). However, in 10 to 30% of cases the malignant B cells fail to express detectable Ig on their cell membrane or in their cytoplasm (1-7). Lack of Ig expression may be caused by aberrations at the DNA level (e.g. non-functionally rearranged Ig genes), at the RNA level (truncated Ig transcripts) or at the protein level (inappropriate protein processing) (1). In a recent report on $SmIg^-$ B-NHL the defective Ig heavy (IgH) chain production could be explained (in 11 out of 19 cases) by inactivation of both IgH genes due to translocation of one allele in combination with deletion or defective rearrangement of the other allele (8).

In 20 to 30% of B-NHL also loss of one or more pan-B cell antigens is found, which leads to abnormalities in marker expression (2,3,5). Although it has been suggested that such abnormalities can be used as diagnostic criterion for lymphoid malignancies (2,5), defining monoclonality may be of utmost importance for making a definite diagnosis in some cases of $SmIg^-$ B-NHL or "null cell" lymphoma (i.e. NHL without obvious B or T cell phenotype).

Virtually all $SmIg^-$ B-NHL have clonal rearrangements of their IgH and/or IgL genes (1,4,6-10). Also plasmacytomas without IgH chain expression ("IgL chain disease") appear to have at least one rearranged IgH gene allele in addition to the IgL gene rearrangements (Figure 1). Also most "null cell" NHL appear to have rearranged IgH and/or IgL genes (without rearranged TcR-β genes), which indicates that they most probably belong to the B cell lineage (11). Therefore analysis of IgH genes (and IgL genes, if necessary) can give valuable diagnostic information concerning clonality and lineage of NHL.

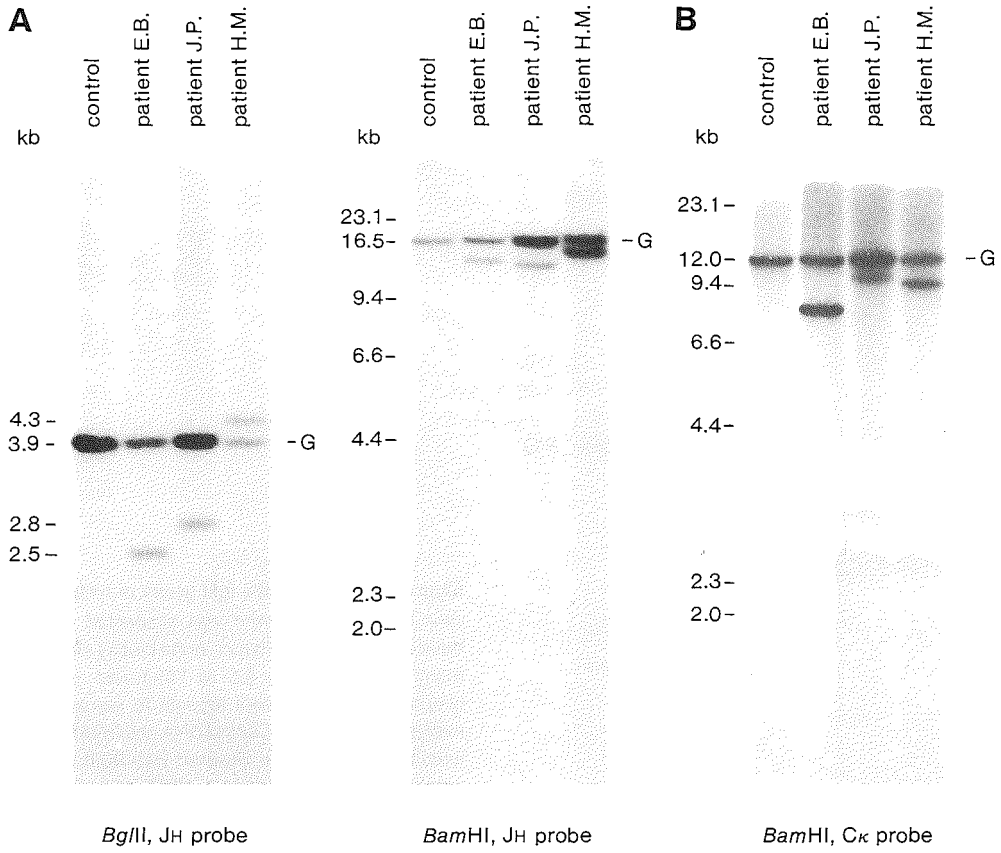


Figure 1. Analysis of the IgH and Igκ genes in BM samples from three patients with "IgL chain disease" (plasmacytoma without IgH chain expression). The malignant plasma cells expressed Igκ in all three patients. The percentages $Ig\kappa^+$ plasma cells were as follows: patient EB: 50%; patient JP: 30%; patient HM: 60%. The DNA samples were digested with *Bgl*II or *Bam*HI, size separated and blotted to nylon membrane filters, which were hybridized with a ^{32}P-labeled JH probe (**A**) or a ^{32}P-labeled Cκ probe (**B**). In all three patients one clonally-rearranged IgH gene band and one clonally-rearranged Igκ gene band were found.

T-cell-rich B cell lymphoma: pseudo T cell lymphoma

Occasionally lymphomas are encountered in which there is a predominance of T cells, suggesting that it concerns a T cell neoplasm on histomorphological and immunophenotypic grounds (12-15). Jaffe and colleagues cautioned for the occurrence of *pseudo-peripheral T cell lymphomas* and pointed to the fact that a small population of monoclonal B lymphocytes may be admixed with reactive T lymphocytes (13,14). These T lymphocytes may comprise up to 75% or more of the total cell number. Especially in patients with follicular lymphomas this may lead to diagnostic problems (14). Southern blot analysis of Ig and TcR genes will generally lead to the appropriate diagnosis (12-17). Figure 2 illustrates the detection of a small population of B-NHL cells in a lymph node with many normal (polyclonal) T and B lymphocytes.

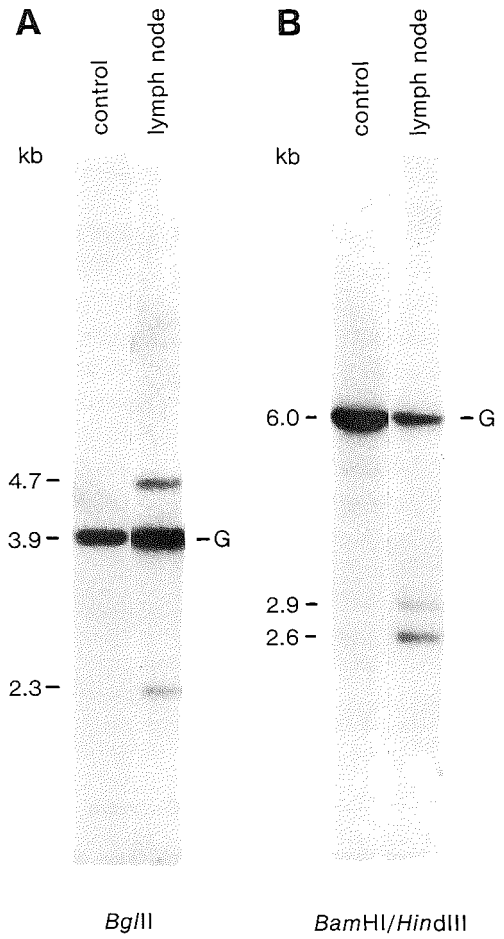


Figure 2. Analysis of the IgH genes of lymph node cells from a patient who was suspected to have developed a NHL. The lymph node biopsy contained many T lymphocytes (60 to 70%) and ~30% B lymphocytes, which were thought to represent the malignant lymphoma. Control DNA and DNA from the lymph node cells were digested with *Bgl*II (**A**) or a combination of *Bam*HI and *Hin*dIII (**B**), size separated and blotted to nylon membrane filters which were hybridized to a ^{32}P-labeled JH probe. In both digests two rearranged bands were detected; the density of these bands corresponds to the size of the B cell population in the lymph node, indicating that it concerned clonal B lymphocytes.

Peripheral T cell lymphoma

Immunologic markers for detection of clonality in T cell proliferations are not available. Nevertheless immature (TdT^+) T cell malignancies such as T cell acute lymphoblastic leukemia (T-ALL) and lymphoblastic T-NHL can easily be detected by use of T cell marker/TdT double immunofluorescence (IF) stainings (18,19). This is based on the fact that TdT^+ T cells normally only occur in the thymus and not in extrathymic locations such as bone marrow (BM), peripheral blood (PB) or lymph nodes (18,20). Even low numbers of malignant TdT^+ T cells (0.01 to 0.001%) are easily detectable by use of the double IF staining technique (21,22; see Chapter 5.3). For mature (TdT^-) T cell malignancies no such marker combination is available. Furthermore, disturbed expression of T cell markers, especially the loss of one or more pan-T cell antigens, is frequently seen in peripheral T-NHL (2,5,23). Like in B-NHL, such marker abnormalities may be useful in the diagnosis of peripheral T-NHL (2,5,23).

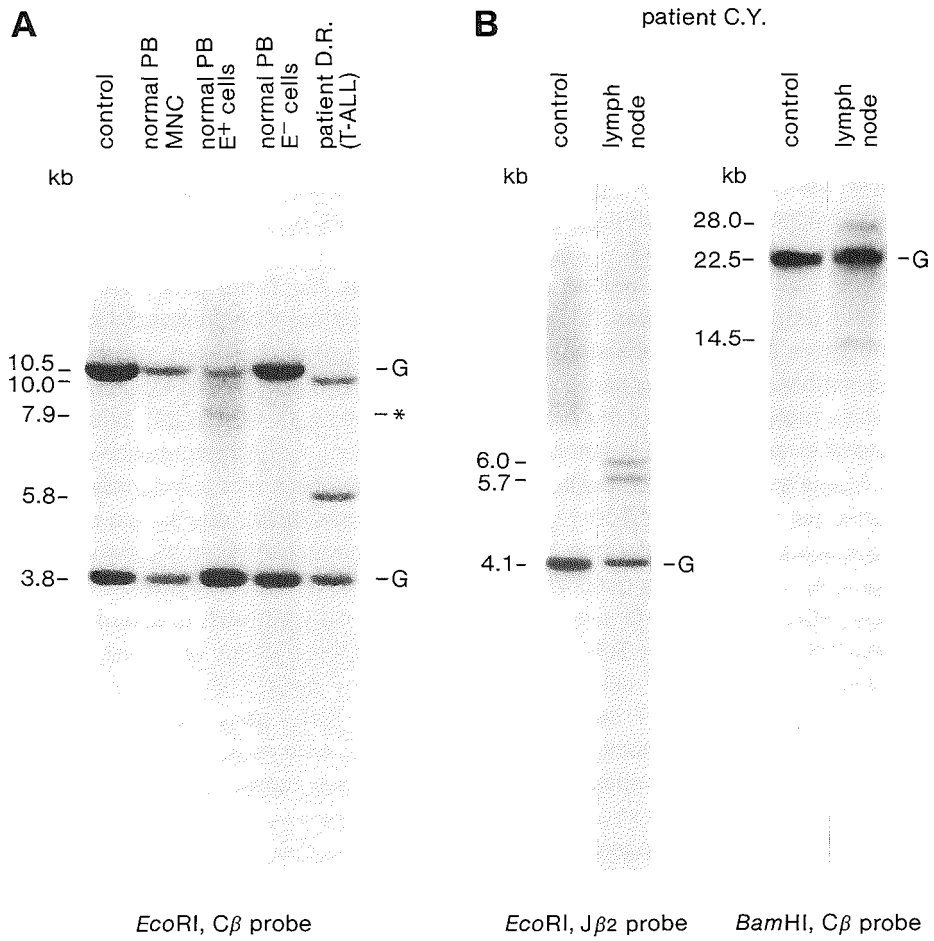


Figure 3. Analysis of the TcR-β genes of normal PB cells, E rosette separated PB cell fractions, a T-ALL cell sample and a lymph node biopsy from a T-NHL patient. Control DNA and DNA from the mentioned cell samples were digested with *Eco*RI or *Bam*HI, size separated in an 0.7% agarose gel and blotted to nylon membrane filters, which were hybridized to a ^{32}P-labeled Cβ probe or a ^{32}P-labeled Jβ2 probe. **A**: *Eco*RI filter hybridized with a Cβ probe. In the E rosette positive (E^+) cell fraction (>95% T lymphocytes) the 10.8 kb band is of markedly reduced density as compared to the E rosette negative (E^-) cell fraction (<5% T lymphocytes). In the E^+ lane no clonally-rearranged bands are visible, but a vague background is present, which probably represents many differently-rearranged bands derived from the polyclonal T lymphocytes. The 7.9 kb band (*) is due to partial digestion (see Chapter 3.3). Only in the lane of the T-ALL sample two clonally-rearranged bands are visible. **B**: *Eco*RI and *Bam*HI digested DNA from the lymph node biopsy of the T-NHL patient, analyzed with the Cβ probe and Jβ2 probe. In both digests two rearranged bands are visible in addition to a strong germline band (G), indicating that a clonal T cell population is present between many lymph node cells with germline TcR-β genes.

Still, in a part of peripheral T cell proliferations it is difficult to discriminate between a T-NHL and a reactive polyclonal T cell proliferation. In most of these cases analysis of the TcR-β genes will resolve this diagnostic problem (24-27).

Analysis of the TcR-β genes of non-T cells by use of *Eco*RI digests and the Cβ probe reveals two germline bands of comparable intensity: 10.5 kb and 3.8 kb (Figure 3A). In polyclonal T cells the intensity of the 10.5 kb band is substantially reduced and no rearranged bands are detectable (also not by use of other digests and other TcR-β probes), indicating that polyclonal rearrangements have occurred on one or both TcR-β gene alleles. If the T cell proliferation concerns a T-NHL, generally one or two rearranged bands are detectable by appropriate analysis of the TcR-β genes (Figures 3B and 4). Detailed information about TcR-β genes is given in Chapter 3.3.

In some $CD3^-$ T-NHL as well as some $CD3^+$ T-NHL the TcR-β genes are in germline configuration (26). In such cases TcR-γ and TcR-δ genes should be analyzed. However, one should be aware that the combinatorial repertoire of both genes is limited, which results in a restricted number of rearranged bands (28-35). Furthermore, recent studies indicate that TcR-$\gamma\delta$ cells exhibit a preferential use of the available V and J gene segments (36-38). Both the limited combinatorial diversity and the preferential V-J gene use hamper the interpretation of the TcR-γ and TcR-δ gene analyses. Especially in case of T cell proliferations with polyclonally rearranged TcR-β genes but "monoclonal" TcR-γ gene rearrangements (39,40), one should consider the possibility that a part of the polyclonal T cells by chance have rearranged the same Vγ and Jγ gene segments, although they generally differ in their Vγ-Jγ junctional regions.

In case of $CD3^+$ T cell proliferations of unknown origin it is worthwhile to determine whether it concerns TcR-$\alpha\beta^+$ T cells or TcR-$\gamma\delta^+$ T cells, because the absence of clonally rearranged TcR-β genes in a TcR-$\alpha\beta^+$ T cell proliferation proves the polyclonality of the proliferation. Furthermore, most TcR-$\alpha\beta^+$ T cells have deleted both TcR-δ gene alleles. Discrimination between polyclonality and monoclonality may be difficult in proliferations of TcR-$\gamma\delta^+$ T cells with germline TcR-β genes. Strictly speaking, monoclonality in such cases can only be determined by proving that the Vγ-Jγ and Vδ-Jδ junctional regions of the involved T cells are identical.

Although TcR gene rearrangements occur in the great majority of T cell malignancies (see Chapter 6.2), the absence of TcR-β and TcR-γ gene rearrangements in a subgroup of peripheral T-NHL has been described (41,42). Therefore in T-NHL TcR gene analysis may not always be helpful in determining clonality (41,42).

Cutaneous lymphoma

It is often difficult to distinguish cutaneous lymphomas from reactive lymphoproliferations on histologic grounds. For instance, early patch stages of mycosis fungoides (MF) can closely resemble some forms of chronic dermatitides (43). Therefore it has been attempted to use additional techniques for evaluation of cutaneous lymphoid infiltrates.

Immunohistology is generally able to distinguish primary or secondary cutaneous B cell lymphomas from benign cutaneous lymphoid hyperplasia (44). In the latter lesions usually normal follicular B and T cell compartmentalization is found (like in nodal hyperplasia) in addition to polyclonal expression of IgL chains, while in most cases of cutaneous B cell lymphoma IgL chain restriction can be used as diagnostic marker (44). If no IgH or IgL chain expression is demonstrable in a cutaneous B cell lymphoproliferative disorder, analysis of IgH genes will be of major value in assessing the diagnostic problem (44).

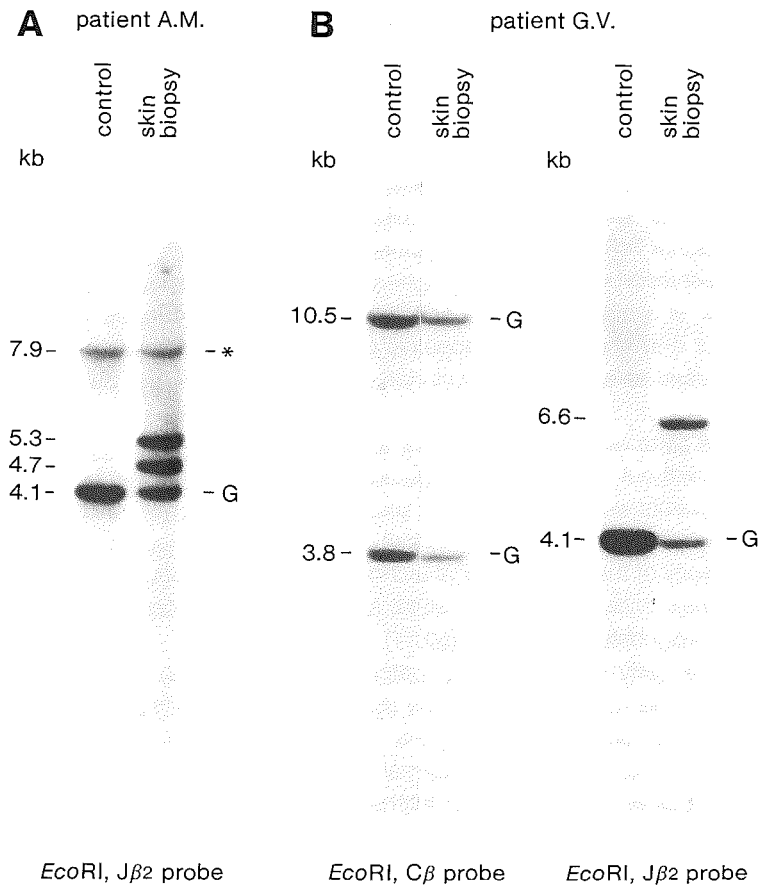


Figure 4. Analysis of the TcR-β genes of skin biopsies from two patients with a T cell infiltrate of unknown origin. Control DNA and DNA from the two skin biopsies were digested with *Eco*RI, size separated and blotted to nylon membrane filters, which were hybridized with ^{32}P-labeled Cβ and Jβ2 probes. **A**: *Eco*RI filter hybridized with the Jβ2 probe. Two rearranged bands were visible in the skin biopsy of patient AM, indicating the presence of a clonal T cell population with rearrangements to the Cβ2 gene locus on both alleles. The 7.9 kb band (*) is due to partial digestion (see Chapter 3.3). **B**: *Eco*RI filter hybridized with the Cβ probe and subsequently with the Jβ2 probe. One rearranged band was visible with the Jβ2 probe, while no rearrangements were detectable with the Cβ probe. This indicates that a clonal T cell population with one rearranged TcR-β gene allele was present in the skin biopsy of patient GV. Both patient AM and patient GV were diagnosed to suffer from mycosis fungoides.

The cutaneous T cell lymphomas in Western countries are mainly represented by MF and Sézary syndrome (SS), which are two closely related disorders (45). MF generally begins with chronic cutaneous patches evolving into plaques and tumor nodules, whereas erythroderma with extreme itching and rapid involvement of blood, lymph nodes and viscera are generally prominent in SS patients (45). Also in MF patients lymph nodes or viscera become often involved (45). Common features in MF and SS are the neoplastic Sézary cells with their cerebriform nuclei and T helper cell phenotype ($CD2^+$, $CD3^+$, $CD4^+$, $CD8^-$) and strong tendency of

the neoplastic cells to migrate into the epidermis (44,45). It has now widely been accepted that SS is a leukemic variant of MF (45). Immunohistology does not represent an easy tool for a definitive diagnosis of MF/SS as many immunophenotypic findings present in MF/SS can also be seen in benign inflammatory dermatoses (44). However, all studies on tumor lesions of MF patients and blood samples of SS patients revealed clonally rearranged TcR-β genes (24-27,43,46) (Figure 4). In addition, in lymph nodes with histologically documented MF involvement as well as in most lymph nodes that histologically showed only dermatopathic lymphadenopathy clonal TcR-β gene rearrangements have been found (27,46,47). The latter is in line with the observation that palpable lymphadenopathy in a MF patient is a poor prognostic sign, regardless of whether the enlarged lymph node was infiltrated by morphologically abnormal cells or only showed dermatopathic changes (48,49). Therefore TcR gene analysis cannot only be used as an additional criterion for diagnosing MF/SS, but also for staging of the disease.

Clonal TcR-β gene rearrangements have also been found in blood samples from several MF/SS patients without morphologically detectable Sézary cells, whereas in other patients clonal rearrangements were not detected in blood samples with small numbers of Sézary cells (50). The latter may be due to the detection limit of routinely performed Southern blotting (~5%) or to the aspecificity of the morphological techniques. These data suggest that both methods should be used next to each other for analysis of blood samples from MF/SS patients in order to obtain better prognostic information (50,51).

Skin lesions of early MF stages (e.g. patch or plaque lesions) appear to have germline TcR-β genes (47). This may be explained by assuming that the infiltrating T cells in these skin lesions are polyclonal rather than monoclonal, i.e. that they are non-malignant. An alternative possibility is that the percentages of abnormal T cells are too low (<5%) to be detectable by routine Southern blot analysis. Whatever interpretation is correct, these findings may explain the indolent course and favorable prognosis seen in most patients with early MF stages (52).

In Japan and the Caribbean, adult T cell leukemia-lymphoma (ATLL) represents a major cause of cutaneous nodular T cell lymphoma (53,54). ATLL cells are positive for human T cell leukemia virus I (HTLV-I) proviral DNA and generally express the CD2, CD3, CD4 and CD25 (IL-2 receptor) antigens (55). In a recent study on a series of 30 ATLL patients it was demonstrated that all ATLL had clonally rearranged TcR-β genes and that in all but one patient also the TcR-γ genes were clonally rearranged (55).

Clonality is equivalent to malignancy in most cutaneous T cell proliferations but several clinically-benign skin lesions can also contain clonal T cell populations: lymphomatoid papulosis (56,57), pityriasis lichenoides et varioliformis acuta (58), granulomatous slack skin (59) and pagetoid reticulosis (60)(see below). Although these skin disorders are not overtly lymphomatous at presentation, they have sometimes been associated with development of lymphoma. Therefore long-term follow-up of patients with a clinically-benign cutaneous T cell proliferation of clonal origin is advisable.

CLONALITY DOES NOT ALWAYS IMPLY MALIGNANCY

Although malignancies are clonal cell proliferations, conversely it is not correct to state that each clonal proliferation represents a malignancy. Benign monoclonal gammapathy and T-γ

TABLE 2. Clinically-benign clonal lymphoproliferations which may evolve into a lymphoid malignancy.

Disease state	Possible evolution into a lymphoid malignancy[a]	References
CLONAL B CELL PROLIFERATIONS		
Benign monoclonal gammapathies		
- in immunodeficient patients (0.1-10 g/l)[b]	–/±	63
- in immunocompetent individuals (3-30 g/l)[b]	+ (10-30%)	67,74
EBV-associated lymphoproliferations		
- in primary immunodeficiencies	+	75,76
- in transplant patients	+	77-83
- in AIDS patients	+	84,85,92
Systemic Castleman's disease	?	96
Extranodal lymphoid hyperplasias		
- ocular adnexa	+	97,98
- lung or gastrointestinal tract	+	99-101,105
- Sjögren's syndrome	+	102,106-108
- Hashimoto's thyroiditis	± (<1%)	103,104
CLONAL T CELL PROLIFERATIONS		
CD8/T-γ lymphocytosis	±	62,111-142
Clinically-benign cutaneous T cell proliferations		
- lymphomatoid papulosis	+ (10-20%)	148
- pityriasis lichenoides et varioliformis acuta	+	58,149
- granulomatous slack skin	+ ?	59
- pagetoid reticulosis	+ ?	60
T cell proliferations in immunodeficient patients	?	92,150,151
Angioimmunoblastic lymphadenopathy with dysproteinemia	+ (20%)	152-156
T cell proliferations in autoimmune diseases	–	171-173

a. Used symbols: –, no evolution into malignancy; ±, rare evolution into malignancy; +, evolution into malignancy may occur in part of the patients; ?, unknown whether evolution into a malignancy may occur.
b. Concentration of homogeneous Ig component in serum.

lymphocytosis are classical examples of clonal clinically-benign proliferations of B and T cells, respectively (61-63). Clonal proliferations of B or T cells have also been found in benign lymphoproliferative diseases with increased susceptibility to the development of a malignant lymphoma (56,57,64-66). Most of them are summarized in Table 2. Especially patients with a primary immunodeficiency or a secondary immunodeficiency (e.g. transplant patients) are prone

to the development of a clonal clinically-benign lymphoproliferative disease, which may transform into a malignancy in some of them.

Benign monoclonal gammapathy

Monoclonal gammapathies were initially only seen in serum of patients with multiple myeloma or other monoclonal B cell proliferative diseases (61,63,67). In these diseases the serum concentration of the monoclonal Ig component is generally higher than 30 g/l (67). With the introduction of more sensitive techniques, monoclonal gammapathies were also found in sera of patients with other clinical conditions i.e. without evidence of multiple myeloma, M. Waldenström, B-NHL or related disorders (61,63). These monoclonal gammapathies of undetermined significance are generally called benign monoclonal gammapathies (61,63,67).

Benign monoclonal gammapathies occur in high frequencies (50 to 100%) in patients with a primary immunodeficiency with predominantly T cell-mediated deficiency as well as in patients with a secondary immunodeficiency such as due to a viral infection (63), bone marrow transplantation (63,68) or renal transplantation (69,70). Also in patients with a hematologic malignancy, autoimmune disease or severe aplastic anemia benign monoclonal gammapathy may occur (63). The benign monoclonal gammapathies in these disease states consist of single or multiple homogeneous Ig components and generally have a serum concentration of 0.1 to 10 g/l (63). Follow-up analyses revealed that most of these monoclonal gammapathies are transient (63,69). There is strong evidence that the development of these transient benign monoclonal gammapathies is due to a disturbance in regulatory T cell functions, especially in case of immunodeficient patients (61,63). This is supported by animal experiments (71,72). However, also healthy individuals may develop benign monoclonal gammapathies (70).

The incidence of benign monoclonal gammapathies in healthy individuals increases with aging and can be as high as 10% in elderly of >80 y (73) or even over 70% in elderly of >95 y (70), depending on the sensitivity of the technique. This suggests that the regulatory T cell functions become increasingly impaired during aging (61,69).

A long-term follow-up study on benign monoclonal gammapathies (serum concentrations: 3-32 g/l) in 241 patients (96% older than 40 y; median age 64 y) revealed that multiple myeloma, M. Waldenström or another malignant lymphoproliferative process developed in 53 patients (actuarial rate of 17% at ten years of follow-up and 33% at twenty years of follow-up) (67,74). So far, there is no reliable technique for absolute discrimination between a patient with benign monoclonal gammapathy and one who will subsequently develop multiple myeloma or another malignant B cell disease (67). The serum concentration of the homogeneous Ig component at first recognition may be informative, since concentrations higher than 30 g/l are generally associated with an overt malignancy. However, the most reliable means of differentiating a benign from a malignant course is the serial measurement of the homogeneous Ig component in the serum together with periodic reevaluation of clinical and laboratory features (67).

Epstein-Barr virus (EBV)-related lymphoproliferative disorders in patients with primary or secondary immunodeficiency

EBV-related illness ranging from fulminant mononucleosis and polyclonal B cell hyperplasia to malignant B cell lymphoma is frequently seen in patients with primary immunodeficiency (75,76) as well as in patients with secondary immunodeficiency, resulting from immune suppressive therapy in allograft transplantation (77-83) or from acquired immunodeficiency syndrome (AIDS) (84,85). EBV is a DNA virus of the herpes family and has a unique tropism for B lymphocytes and an inherent ability to transform and immortalize the host cell (64,65).

The EBV-related B cell lymphoproliferative disorders in immunodeficient patients frequently occur outside lymph nodes, e.g. in brain or soft tissues (64,65). They have a high tendency to progress and often have a fatal outcome (64,65). The incidence of B cell lymphoproliferations in immunodeficient patients appears to depend on the type of immunodeficiency. They are relatively frequently seen in primary immunodeficiency syndromes such as ataxia telangiectasia and Wiskott-Aldrich syndrome (86,87). In transplant recipients the overall incidence is ~0.6% for bone marrow transplant (BMT) patients (82), 1 to 2.5% for renal transplant patients (79,80,88), ~2.3% for liver transplant patients (79), and 4.9 to 6.3% for heart transplant patients (79,80,89). The frequency of abnormal B cell proliferations in AIDS patients is estimated to be 3 to 4% (65). In a large study on BMT patients it was found that T cell depletion of the donor marrow as well as administration of CD3 antibodies for treatment of graft-versus-host disease are high risk factors for developing EBV-related lymphoproliferations (82). These data together suggest that the incidence of the lymphoproliferative diseases is related to the degree of T cell deficiency.

Despite the high mortality of the EBV-related B cell lymphoproliferations, several investigators have reported that many of these B cell proliferations are polyclonal when tested with anti-Igκ and anti-Igλ antibodies (64,65,79,82,90). In some cases a monoclonal B cell population was identified, often not earlier than in the final stage of the disease (64,65,79,82,90, 91). In other cases no Ig expression could be demonstrated (65,79,89). Remarkably, in several of the immunophenotypic-"polyclonal" lesions of renal transplant patients clonal cytogenetic aberrations have been found (64), while in most immunophenotypic-"polyclonal" lesions of BMT patients clonal IgH gene rearrangements were detected (82). Also in 18% of the enlarged lymph nodes from patients with AIDS-related lymphadenopathy syndrome (LAS) clonal Ig gene rearrangements were found, although they are commonly considered to represent benign polyclonal lymphoid proliferations (85).

Southern blot analysis of the Ig genes of B-NHL in a series of transplant patients and a series of AIDS patients revealed the presence of clonal IgH and/or IgL gene rearrangements (82,83,85,89,92). In several cardiac transplant patients multiple lesions could be studied, which revealed that the Ig gene rearrangements could differ between the lesions within a single patient (93). This led the investigators to suggest that lymphoproliferative disorders in transplant patients are multiclonal diseases (93). Interestingly, in some lesions of AIDS patients more than two rearranged IgH gene bands of different density were seen, indicating that more than one B cell clone occurred in these lesions (85,92). Furthermore, in the majority of B cell lymphomas from AIDS patients a rearrangement of the c-*myc* oncogene and/or a Burkitt

translocation was found, which indicates that it concerned malignant processes (85). In the clinically-benign LAS lesions of AIDS patients no such c-*myc* gene rearrangements were detectable, although in some of them clonally-rearranged IgH gene bands were found (85).

All data together are in line with the hypothesis that EBV is an initiator of a multistep process that may ultimately lead to the development of a malignant B cell lymphoma (64,65,94). Especially patients with a severe suppression of T cell function seem to be at risk to develop such malignant lymphomas. EBV infection results in a polyclonal activation and proliferation of B lymphocytes. In the absence of sufficient T cell immunity an uncontrolled proliferation of "normal" B lymphocytes occurs, which may lead to oligoclonal or even monoclonal outgrowth of "normal" B cells due to growth advantage. During this uncontrolled proliferation malignant transformation may occur (e.g. a chromosomal aberration involving one or more oncogenes), leading to the development of a malignant B cell lymphoma (64,65,94), or multiple lymphomas (85,92,93).

Oligoclonal or monoclonal B cell populations may remain undetectable by use of anti-IgL chain antibodies, because many polyclonal B cells may still be present and/or because the malignant cells may not express Ig molecules due to damage of one of the Ig genes (1,8). However, the occurrence of oligoclonal or monoclonal B cell populations in lymphoproliferative lesions of immunodeficient patients can easily be detected by use of Southern blot analysis of Ig genes, if the clonal B cell population represents more than 5% of the cells (82,83,85,89,92, 93). Whether the detected oligoclonal or monoclonal B cell populations are already malignantly-transformed, can only be investigated by additional studies, such as cytogenetic analysis or Southern blot analysis of the relevant oncogenes (85).

In transplant patients with predominantly regional lymphoproliferative disease, the lesions appear to regress upon reduction or discontinuation of immunosuppression, often without subsequent rejection of the graft (65,79). This suggests that the lymphoproliferative lesions in such patients were not yet malignantly-transformed, although oligoclonal or monoclonal B cell populations may already have been present. Systemic cytotoxic treatment of EBV-related lymphoproliferations in immunodeficient patients carries the potential for further immunosuppression and should be reserved for patients with a progressive disease, i.e. with malignantly-transformed lesions (65). However, the results of conventional cytotoxic treatment are disappointing and mortality remains high (65).

In conclusion, the EBV-related B cell lymphoproliferations can be regarded as basically benign proliferations, which have a high tendency to malignant transformation in immunodeficient patients with suppressed T cell functions. An appropriate diagnosis and early withdrawal of immunosuppressive treatment in transplant patients before malignant transformation has occurred, will probably decrease the mortality rate. Southern blot analysis of the Ig genes in the lymphoproliferative lesions may be helpful to discriminate between polyclonal lesions and lesions with oligoclonal or monoclonal B cell populations, which are difficult to detect with anti-IgL chain antibodies. However, for discrimination between "normal" (oligo)clonal B cell populations and malignant B cell populations additional investigations are needed, such as cytogenetics and Southern blot analysis of the relevant oncogenes.

Castleman's disease

Castleman's disease is an uncommon lymphoproliferative disorder, which is morphologically and clinically heterogeneous. Both a localized benign variant and a more aggressive form with systemic manifestations have been described (95).

Two types of local variants are known: a hyaline vascular type and a plasma cell type with sheets of polyclonal plasma cells. These local variants of Castleman's disease are self-limiting processes, which are often localized in central lymph nodes, although also extranodal locations occur (95). Only mild systemic manifestations such as fever, anemia and hypergammaglobulinemia are seen in these patients. The disease is curable with local therapy (e.g. surgery), which generally also results in the disappearance of the systemic manifestations (95).

The plasma cell type can also occur as a systemic multicentric variant of Castleman's disease. This variant is characterized by extensive peripheral lymphadenopathy, hepatosplenomegaly, more severe clinical manifestations and an aggressive clinical course. The systemic variant is associated with infectious complications and an increased risk for developing neoplasias such as Kaposi's sarcoma and B cell malignancies (95,96).

In a recent study clonal IgH gene rearrangements were found in the lymphoproliferative lesions of three of the four patients with systemic Castleman's disease, while in four patients with localized disease only germline bands were found (96). Interestingly, in two of the three patients with clonal IgH gene rearrangements copies of EBV genome were detected.

These findings suggest that the lesions in systemic Castleman's disease resemble the EBV^+ lymphoproliferations in immunodeficient patients (96). This is further supported by the fact that several features of systemic Castleman's disease are frequently seen in immunodeficient states, e.g. frequent infections, Kaposi's sarcoma and morphological similarities to lymph node lesions of AIDS patients (95,96).

Extranodal noncutaneous lymphoid hyperplasia ("pseudolymphoma")

About 10 to 25% of NHL are located outside the major lymphoid tissue-bearing sites. The most common extranodal sites are the gastrointestinal tract, skin and soft tissues, ocular adnexa, respiratory tract, and salivary glands, but extranodal NHL can arise in any conceivable anatomic site (97). A heterogeneous group of morphologically-benign lymphoid hyperplasias also occur in identical extranodal sites (97,98). They generally consist of small lymphocytic proliferations and are often designated "pseudolymphomas". These benign and malignant extranodal lymphoproliferations represent a difficult diagnostic dilemma due to their overlapping clinical and histopathologic features (97,98). They even occur in association with each other, suggesting a pathogenetic relationship (97,98). This especially concerns patients with lymphoid hyperplasias in ocular adnexa, lung or gastrointestinal tract (97-101), patients with Sjögren's syndrome (102) or patients with Hashimoto's thyroiditis (103,104).

Immunologic marker studies, especially determination of restricted IgL chain expression, demonstrated that a large part of the morphologically-benign pseudolymphomas on a solitary

noncutaneous extranodal site are in fact monoclonal B cell proliferations, which generally have a benign clinical course and long survival with only minimal therapeutic intervention (98,105). Only a minority of patients with a morphologically-benign, immunophenotypic-monoclonal lesion appeared to develop systemic malignant lymphoma within 2 to 5 years after initial presentation. However, this also occurred in patients with an immunologic-polyclonal lesion (98,106).

Several recent studies have demonstrated that clonal rearrangements of IgH and/or IgL genes are present in the majority of the histopathologically-benign, immunophenotypic-polyclonal lymphoid hyperplasias occurring in solitary extranodal sites. This concerned lymphoid lesions in ocular adnexa, diffuse lymphoid hyperplasias of the breast, benign lymphoepithelial lesions in salivary glands (often found in patients with Sjögren's syndrome) and the lymphocytic infiltration in the thyroid gland of a patient with Hashimoto's thyroiditis (97,107-110). The patterns of Ig gene rearrangements varied from one or two distinct bands to multiple faint bands, which indicates the presence of monoclonal or oligoclonal B cell populations respectively (97,107-110). In a particular patient with Sjögren's syndrome two distinct benign lymphoepithelial lesions were removed two years apart; the rearrangement patterns of the IgH as well as IgL genes in these two lesions were entirely different (108). So far, in the cases studied, no clonal TcR-β gene rearrangements were found (97,110) and no EBV genomic sequences were detected (97). These reports indicate that the majority of morphologically-benign and immunophenotypic-polyclonal extranodal noncutaneous lymphoid hyperplasias contain "occult" (oligo)clonal B cell populations.

In three out of sixteen solitary lymphoid hyperplasias with clonally-rearranged Ig genes also rearrangements in the *bcl*-1 or *bcl*-2 genes were found, which suggests that a malignant transformation had occurred in these patients. Nevertheless, after local treatment (irradiation) neither local recurrence nor systemic disease were seen in these patients during follow-up periods of 4, 29 and 30 months (97). However, two other patients with immunophenotypic-polyclonal, but genotypic-monoclonal lymphoid hyperplasias developed overt malignant lymphoma, despite conservative surgical treatment (without irradiation) of the primary lesion (97).

All data together suggest that the lymphoproliferative lesions in the various extranodal noncutaneous sites represent a continuous and progressive spectrum from true polyclonal B cell proliferations to malignant B cell lymphomas (97). Initially, small (oligo)clonal B cell populations may develop as a consequence of local or general defects in the mechanisms that normally regulate B cell proliferation. These clonal B cell expansions may become susceptible to genetic alterations (e.g. rearrangements of the *bcl*-1 or *bcl*-2 genes or other till now undetermined genes), which lead to malignant transformation (97). This is in line with the increased incidence of B-NHL in patients with lymphoid hyperplasia in ocular adnexa, lung or gastrointestinal tract, patients with Sjögren's syndrome and patients with Hashimoto's thyroiditis (97-104).

It has been suggested that the best therapeutic approach for patients with a solitary extranodal lymphoid hyperplasia probably is irradiation of the local disease site, followed by a careful monitoring for lymphoid proliferations (97). Immunophenotyping and analysis of Ig genes are important to support the diagnosis. Detection of rearrangements in *bcl*-1 or *bcl*-2 genes or other markers of malignant transformation, which may become available in the future, will probably allow us to distinguish between benign and malignant monoclonal lymphoproliferations in extranodal sites (97).

Chronic T cell lymphocytosis: T cell chronic lymphocytic leukemia, T-γ lymphocytosis, CD8 lymphocytosis and granular lymphocyte proliferative disorder

In the literature mature T cell proliferative disorders with a chronic clinical course have been described under a variety of names, such as chronic T cell lymphocytosis, T cell chronic lymphocytic leukemia (T-CLL), T-γ lymphocytosis, CD8 lymphocytosis, natural killer cell leukemia, large granular lymphocyte leukemia and granular lymphocyte proliferative disorders (62,111-113). Most probably all these chronic T cell proliferative disorders form one disease entity, which predominantly occurs in males of older age (median age of 55-65 y) and is characterized by a moderate blood T lymphocytosis (ranging from 5,000/μl to 20,000/μl) with T lymphocyte infiltration of the BM and often the spleen (62,111-113). While in most patients chronic T cell lymphocytosis is not an aggressive disease, the patients are often neutropenic and have recurrent bacterial infections requiring antibiotic therapy. Some patients with a more aggressive disease may benefit from cytotoxic chemotherapy, but most patients do not require such treatment (62,111-113).

The T lymphocytes generally have a granular lymphocyte morphology, express the pan-T cell markers CD2 and/or CD7 and the majority of them are positive for the CD8 molecule and/or the CD16 molecule. The latter represents the Fcγ receptor type III (FcγRIII) (62,111-113).

One of the confusing topics concerning chronic T cell lymphocytoses has been the difficulty in determining whether it is a reactive polyclonal or a neoplastic monoclonal disorder (113). A large series of publications have described that most chronic T cell proliferative disorders have clonally rearranged TcR-β and/or TcR-γ genes (24,25,62,114-142). The data become more clear when the T cell disorders are divided into $CD3^+$ types (~80%) and $CD3^-$ types (~20%) (Table 3).

As far as studied, the majority of the $CD3^+$ chronic T cell lymphocytoses express TcR-αβ and only a minority express TcR-γδ (62,130,141). Most $CD3^+$ types are positive for the CD8 molecule and the CD57 molecule (Leu-7/HNK-1), about half of them express the CD16 molecule and about one-fourth is positive for the CD56 molecule (neural cell adhesion molecule; NCAM) (Table 3). They generally exhibit antibody dependent cytotoxicity (ADCC) activity, but most of them lack NK activity (62,111-113). The far majority of the $CD3^+$ chronic T cell lymphocytoses have clonally rearranged TcR-β and/or TcR-γ genes (Table 3 and Figure 5). Some $CD3^+$ lymphocytoses with germline TcR-β genes appeared to have clonally rearranged TcR-γ genes and therefore probably expressed TcR-γδ (133,141). It may well be that all $CD3^+$ lymphocytoses with germline TcR-β genes are in fact clonal TcR-$\gamma\delta^+$ T cell lymphocytoses (Table 3).

The $CD3^-$ chronic T cell lymphocytoses generally have the $CD4^-/CD8^-$ phenotype, although about one-fourth may express the CD8 molecule; the majority express the CD16 molecule as well as the CD56 molecule and half of them is positive for the CD57 molecule (Table 3). Most $CD3^-$ lymphocytoses exhibit both ADCC and NK activity (62,111-113). All $CD3^-$ chronic T cell lymphocytoses appeared to have germline TcR-β and germline TcR-γ genes (Table 3 and Figure 5).

All data together indicate that the $CD3^+$ lymphocytoses are clonal counterparts of normal cytotoxic T lymphocytes (62,113), while the $CD3^-$ T cell lymphocytoses with germline TcR

TABLE 3. Summary of data from the literature concerning chronic T cell lymphocytoses, such as T-CLL, T-γ lymphocytosis, CD8 lymphocytosis and large granular lymphocyte proliferations[a].

	$CD3^+$ lymphocytosis	$CD3^-$ lymphocytosis
Relative frequency (within the total group)	~80%	~20%
Neutropenia	majority (90%)	about half of the patients
Cellular functions[b]		
- ADCC activity	majority	majority
- NK activity (non-MHC restricted cytotoxicity)	minority	majority
Immunophenotype		
TcR-αβ	85-90%	–
TcR-γδ	10-15%	–
$CD4^+/CD8^-$	rare (3/67)	– (0/17)
$CD4^-/CD8^+$	majority (58/67)	~25% (4/17)
$CD4^+/CD8^+$	rare (4/67)	rare (1/17)
$CD4^-/CD8^-$	rare (2/67)[c]	majority (12/17)
CD16 $(Fc\gamma RIII)^+$	~55% (27/49)	majority (11/13)
CD56 $(NCAM)^+$	~25% (6/23)	majority (5/5)
CD57 $(Leu\text{-}7/HNK\text{-}1)^+$	~90% (52/58)	~50% (8/15)
TcR gene rearrangements		
- clonal TcR-β genes	84% (63/75)	0% (0/19)
- polyclonal TcR-β genes	9% (7/75)	0% (0/19)
- germline TcR-β genes	7% (5/75)[d]	100% (19/19)
- clonal TcR-γ genes	97% (32/33)[e]	0% (0/8)

a. The immunophenotypic and TcR gene data are derived from publications which report the absence or presence of CD3 expression (refs. 24,25,62,116,117,119-121,123-130,132,133,135,140,141). Several research groups have published more than one manuscript about chronic T cell lymphocytosis. To avoid duplications, we have only included the most informative data of each group by selecting the publications: combination of refs. 116 and 130, leaving out refs. 139 and 142; combination of refs. 121 and 132; ref. 133, leaving out ref. 131; ref. 62, leaving out refs. 134 and 136; ref. 135, leaving out ref. 137.

b. Data from refs. 62, 111-113.

c. Only two out of sixty-seven $CD3^+$ T cell lymphocytoses appeared to have the $CD4^-/CD8^-$ phenotype. Both expressed TcR-γδ (62,141).

d. In three out of five reported $CD3^+$ T cell lymphocytoses with germline TcR-β genes also the TcR-γ genes were analyzed. In all three cases the TcR-γ genes appeared to be clonally rearranged (133,141).

e. In one out of thirty-three $CD3^+$ T cell lymphocytoses the TcR-γ genes seemed to be in germline configuration. However, only the Jγ1.3 probe was used in this patient (123).

genes are counterparts of normal NK cells, which also have germline TcR genes (62,113,130, 142-146).

Like in other clonal clinically-benign lymphoproliferations, it may be important to monitor patients with a chronic T cell lymphocytosis in order to detect clinical evolution or remission of the disease. This monitoring can be supported by immunophenotyping or Southern blot analysis of TcR genes. The latter is important to determine whether a clinical remission of a $CD3^+$ lymphocytosis actually results from or coincides with disappearance of the T cell clone (113).

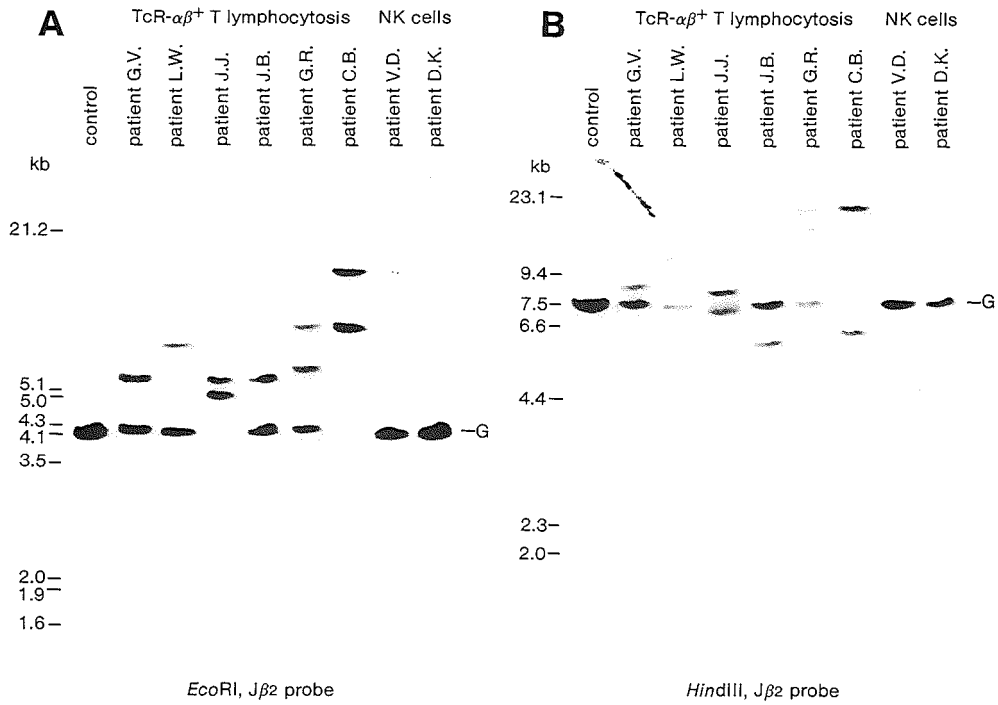


Figure 5. Analysis of the TcR-β genes of PB cells from eight patients with a CD8/T-γ lymphocytosis. Six patients had a TcR-$\alpha\beta^+$ T lymphocytosis, while two patients had a NK cell ($CD3^-$) T lymphocytosis. Control DNA and DNA from PB cells of the eight patients were digested with *Eco*RI (**A**) or *Hin*dIII (**B**), size separated and blotted to nylon membrane filters, which were hybridized with a ^{32}P-labeled Cβ probe (not shown) and a Jβ2 probe. Clonally rearranged TcR-β genes were found in all patients with TcR-$\alpha\beta^+$ T lymphocytosis, whereas in the patients with NK cell lymphocytosis only germline TcR-β genes were observed.

In some patients with rheumatoid arthritis also T cell lymphocytosis, splenomegaly and neutropenia occur. This combination is termed Felty's syndrome (111,125,147). However, the T lymphocytes in blood of patients with Felty's syndrome appeared to be polyclonal instead of monoclonal (125).

Clinically-benign cutaneous T cell proliferations

Several clinically-benign skin lesions appear to contain clonal T cell populations: lymphomatoid papulosis (56,57), pityriasis lichenoides et varioliformis acuta (58), granulomatous slack skin (59) and pagetoid reticulosis (60).

Lymphomatoid papulosis is characterized by the intermittent appearance of papular eruptions of the skin, which eventually ulcerate and later resolve as scars. The disease usually has a benign chronic course, although the histologic appearance of the skin lesions are consistent with that of a malignant neoplasm: the skin infiltrates contain a prominent population

of large atypical Reed-Sternberg-like cells or atypical cells with cerebriform nuclei. Evolution to malignant lymphoma may occur in 10 to 20% of the patients (148). Analysis of the TcR-β genes revealed that in a large part of patients with lymphomatoid papulosis a clonal T cell population is present in their skin lesions (56,57). In one patient three separate skin biopsies were analyzed, which showed varying patterns of TcR-β gene rearrangements (56). This suggests that lymphomatoid papulosis may be multiclonal in origin. However, in another patient the same rearrangement pattern was found in two separate skin lesion biopsies taken eleven months apart (57), which illustrates the indolent course of the disease. Like in other clinically-benign clonal cell proliferations, also lymphomatoid papulosis may develop from a polyclonal T cell proliferation via an oligoclonal stage into a monoclonal T cell lesion, which may transform into a T cell lymphoma in some patients. To prove such a multistep process, prospective studies are needed.

Pityriasis lichenoides et varioliformis acuta (PLEVA) or Mucha-Habermann disease is characterized by extensive recurrent skin eruptions of papules that ulcerate and then heal over a period of weeks. It is believed to be an inflammatory disorder and not a lymphoproliferative process. Nevertheless, in a study on three patients with PLEVA all three patients appeared to have a clonal T cell population in their skin lesions (58). Clinically and histopathologically PLEVA shows several similarities to lymphomatoid papulosis (149), which is supported by the fact that both types of skin lesions can contain clonal T cell populations and that they may be associated with more aggressive neoplasms such as mycosis fungoides (58,149). The principal difference between the two types of skin lesions is that lesions of lymphomatoid papulosis contain numerous large atypical lymphoid cells, which are lacking in PLEVA skin lesions (58,149).

Granulomatous slack skin is a rare disorder, characterized by the slow evolution of bulky erythematous skin folds that have a granulomatous histology and show destruction of dermal elastic tissue (59). The skin lesions contain a dense lymphohistiocytic infiltrate, in which the lymphocytes mainly exhibit the $CD3^+/CD4^+/CD8^-$ immunophenotype. Analysis of the TcR-β genes in the skin lesions of three patients demonstrated the presence of a major clonal T cell population in each case (59). Granulomatous slack skin has several features in common with mycosis fungoides, but granulomatous slack skin shows a marked tendency towards development of flexural lesions and the bulky lesions do not contain the atypical lymphoid cells which are seen in mycosis fungoides. Nevertheless, LeBoit and colleagues concluded that granulomatous slack skin is a slowly progressive lymphoma (59).

Pagetoid reticulosis is a rare skin disorder consisting of solitary or localized, often hyperkeratotic, cutaneous plaques. The involved skin areas show a prominent infiltrate of atypical T cells within a hyperplastic epidermis. Clinically these lesions are indolent (60). However, in a recent publication, it was demonstrated that a clonal T cell population was present in the skin lesions of a patient with localized pagetoid reticulosis (60). Because some patients with disseminated forms of pagetoid reticulosis have died from mycosis fungoides, it may be important to monitor patients with localized disease (60).

Apparently, there exists a broad spectrum of cutaneous T cell lymphoproliferative disorders with various types of clinically-benign disorders at one side and mycosis fungoides and other overt T cell lymphomas at the other side. All these cutaneous T cell disorders exhibit immunogenotypic evidence of oligoclonality or monoclonality and in several patients more than

one of these diseases occur either concurrently or sequentially (60). The above described clinically-benign cutaneous T cell lymphoproliferative disorders are not overtly lymphomatous from their onset, but they have the potential to evolve into more aggressive forms of disease. Therefore, long-term follow-up is indicated in patients with lymphomatoid papulosis, PLEVA, granulomatous slack skin or pagetoid reticulosis.

Monoclonal and oligoclonal T cell proliferations in immunodeficiency

During the last four years we have detected oligoclonal autologous T cell proliferations in two patients (RR and GB) with severe combined immunodeficiency (SCID) as well as a monoclonal T cell proliferation in a kidney transplant patient (JB) during immunosuppression (Figure 6)(150). The T cell proliferation in these three patients concerned a T cell lymphocytosis with the $CD3^+/TcR\text{-}\alpha\beta^+/CD4^-/CD8^+$ immunophenotype. In SCID patient RR also T cell infiltrates in liver and lung were found. The infiltrate in the lung of this SCID patient consisted of only one of the three T cell clones which were present in PB (Figure 6). Despite the (oligo)clonality of the $CD8^+$ T cell proliferations no signs of malignancy were seen in the three immunodeficient patients during a follow-up period of 1 to 4 years. SCID patients RR and GB died from severe infections 2½ y and 1 y after detection of the oligoclonal T cell proliferations, respectively.

A few reports have described the occurrence of clonal T cell proliferations in immunodeficient patients, i.e. some patients with AIDS (92) and a patient with a combined immunodeficiency (CID)(151). In the AIDS patients it concerned monoclonal $CD3^+/CD4^-/CD8^+$ T lymphocytoses, which were interpreted as being chronic lymphocytic leukemias with an indolent course (92). In the CID patient it concerned an oligoclonal $CD3^+/TcR\text{-}\alpha\beta^+/CD4^-/$ $CD8^-$ T lymphocytosis with massive lymphadenopathy and hepatosplenomegaly. Although the oligoclonal T cells in the CID patient had an autologous origin, the massive T cell infiltrates and histologic features of the skin were similar to those in graft-versus-host disease. Therefore cyclosporin therapy was initiated, resulting in a dramatic clinical improvement. Because of the unusual $CD4^-/CD8^-$ phenotype of the majority of the oligoclonal T cells, the authors have suggested a relationship with autoimmune processes (151).

The frequency of (oligo)clonal T cell proliferations in patients with primary or secondary immunodeficiency is probably higher than suggested by publication of only a few reports. This scarcity of reports on this subject may be related to the fact that T cell clonality can only be proven at the DNA level, since no protein markers are available for the detection of clonality in T cell proliferations.

The precise nature of the clinically-benign clonal T cell proliferations in immunodeficiency patients is not yet clear. Do they represent antigen-triggered clonal expansions, are they virally-induced proliferations or are they related to CD8/T-γ lymphocytosis? Like in immunodeficient patients with benign monoclonal gammapathies or EBV-associated (oligo)clonal B cell proliferations (63,71,72,82), failing T cell control might allow inappropriate expansion of a few (generally $CD8^+$) T cell clones. Follow-up studies will elucidate whether these clinically-benign clonal T cell proliferations can transform into overt T cell malignancies.

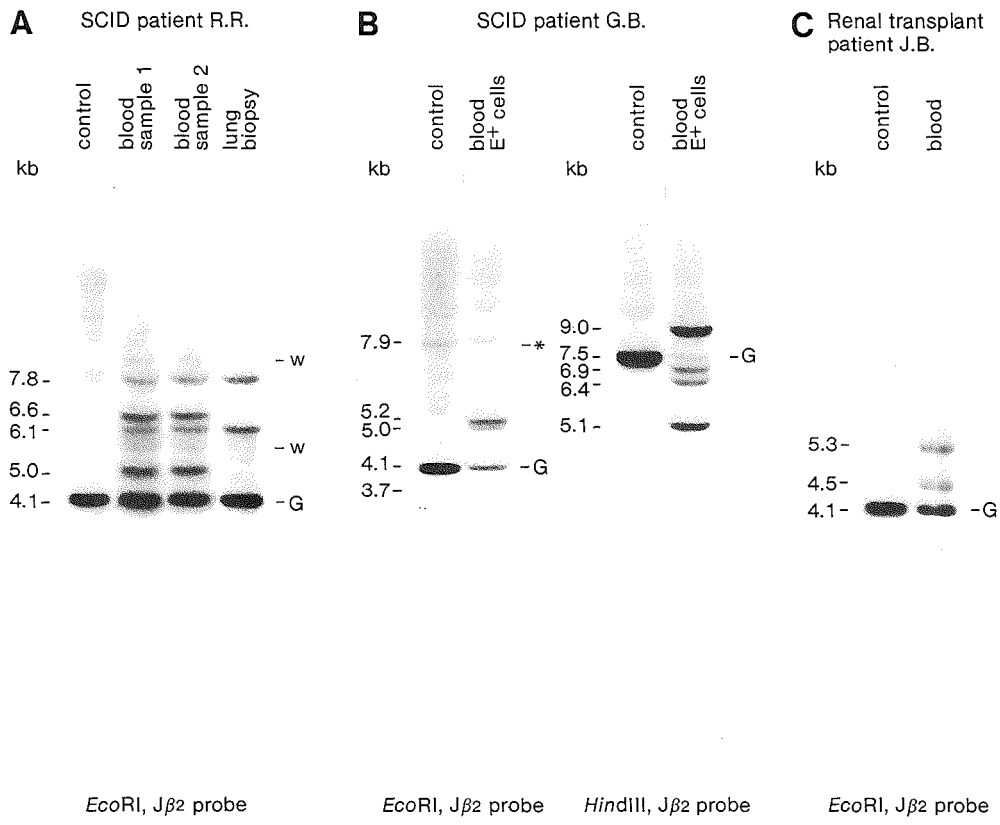


Figure 6. Analysis of the TcR-β genes of cell samples from two SCID patients and a renal transplant patient. Control DNA and DNA from the patient samples were digested with *Eco*RI or *Hin*dIII, size separated and blotted to nylon membrane filters, which were hybridized with the Cβ probe (not shown) or the Jβ2 probe. **A**: *Eco*RI filter with DNA from two PB samples and a lung biopsy of SCID patient RR which was hybridized with the Jβ2 probe. Four distinct rearranged bands and two faint bands were present in the two PB samples. In the lung biopsy only two rearranged bands were detected, which have the same size as two of the distinct rearranged bands in the PB lanes. These data indicate that most probably three T cell clones were present in the PB of this SCID patient and that one of these T cell clones had infiltrated the lung (ref. 150). **B**: *Eco*RI and *Hin*dIII filters with DNA from E rosette positive (E^+) PB cells (>95% T lymphocytes) of SCID patient GB. The filters were hybridized with the Jβ2 probe. In the *Hin*dIII digest two strong and two weak rearranged bands were visible in addition to a faint germline band (G). In the *Eco*RI digest only one strong rearranged band and two faint rearranged bands were present. The band at the germline position probably represents also a rearranged band, which co-migrates with the germline band. The 7.9 kb band (*) in the *Eco*RI digest is due to incomplete digestion (see Chapter 3.3). These data indicate that two T cell clones of different size were present in the PB sample of patient GB. **C**: *Eco*RI filter with DNA from PB cells of kidney transplant patient JB. The filter was hybridized with the Jβ2 probe, which revealed the presence of two rearranged bands in addition to the germline band (G). This indicates that a clonal T cell population was present in the PB of this patient.

Angioimmunoblastic lymphadenopathy with dysproteinemia

Angioimmunoblastic lymphadenopathy with dysproteinemia (AILD) is a systemic disease of unclear etiology, which generally develops at older age (>60 y) (66,152-156). AILD is characterized by generalized lymphadenopathy and in many patients by hepatosplenomegaly and skin rashes (66,152-156). Polyclonal hypergammaglobulinemia is often present and may be associated with a Coombs-positive hemolytic anemia (66,152-156). The clinical picture is consistent with a lymphoma-like syndrome, but histologically AILD resembles a benign lymphoproliferative disorder (66,152-156).

Involved lymph nodes in AILD show an obliteration of the normal architecture by a polymorphic cellular infiltrate consisting of small lymphocytes, immunoblasts and plasma cells together with a proliferation of arborizing small blood vessels (66,152-155). Immunophenotypic studies demonstrated that most lymphoid cells in AILD lymph nodes express T cell markers, but also polyclonal populations of small B lymphocytes and plasma cells may occur (66,154,157).

Despite the "benign" histomorphology, in the majority of AILD patients the clinical course is aggressive with a median survival of less than two years (66,152-156). Most patients die from opportunistic infections (~50%), malignant lymphoma (~20%), other malignancies or unrelated causes (66,152-156). On the other hand, about 25 to 30% of the AILD patients reach spontaneous remission (unrelated to treatment), which illustrates the unpredictable course in AILD (152-156).

The lymphomas in AILD patients may be of B cell origin (154,158-160), but probably most are of T cell origin (66,157,161). To study whether the development of a lymphoma is related to the presence of clonal T or B cell populations in the benign AILD infiltrates, Ig and TcR gene analyses have been performed on biopsies from involved lymph nodes. These analyses revealed that in ~70% of cases clonal Ig and/or TcR gene rearrangements were present (161-166). In most of these cases (~85%) this concerned clonal TcR-β gene rearrangements, indicating the presence of T cell clones (161-166). In some of them (5 to 10%) clonal IgH and/or IgL gene rearrangements were found, which is in line with the presence of B cell clones (162,165). Finally, in some patients (5 to 10%) both clonal Ig and clonal TcR gene rearrangements were observed, which suggests that two different clonal cell populations were present (164-166). The rearranged bands are often faint or have a low density as compared to bands in control samples (163,165). This indicates that the B or T cell clones represent (small) subpopulations of the lymph node infiltrates. It should be noted that the Ig or TcR gene rearrangement patterns can differ between separate lymph node biopsies from a single AILD patient. In addition, clonal Ig and TcR gene rearrangements can emerge or disappear during the course of the disease (165).

Several investigators have suggested that AILD may be a disorder of immunoregulation in which lymphoid cells excessively proliferate, leading to clonal expansion(s) of T lymphocytes and/or B lymphocytes (162,163,165). This immune-dysregulation theory is supported by the observation that many AILD patients have hypergammaglobulinemia in association with autoimmune hemolytic anemia and that approximately half of the patients die from severe infections such as pneumonia and septicemia (152-156).

However, in AILD patients who die from a malignant T or B cell lymphoma, the lymphoma may have been derived from one of the expanded T or B cell clones, due to a genetic error during the repeated cell divisions (165). Indeed clonal cytogenetic aberrations have been detected in AILD lymph nodes (167-170). These may represent secondary events, which account for malignant transformation and selective proliferation of the malignant clone. On the other hand, like in Ig and TcR gene studies, also sequential cytogenetic studies indicate that some clones disappear, while other clones emerge (66,169).

Prospective studies are needed to unravel the role of clonal T and B cell populations in AILD patients. Do they represent pre-malignant proliferations in a multi-step process or (transient) clonal expansions as a consequence of disturbed immune functions?

Clonal T cell populations in autoimmune disease

In several autoimmune disorders T lymphocytes dominate in the local inflammatory infiltrates. TcR-β gene analysis of synovial fluid T lymphocytes from patients with rheumatoid arthritis (RA) showed a dominant rearranged band in three out of eleven RA patients tested (171). Also in two patients with chronic progressive multiple sclerosis (MS) evidence for oligoclonal T cells in cerebrospinal fluid (CSF) was found upon analysis of the TcR-β genes of independent CSF-derived T cell clones (172). In addition, in one of six cases with Hashimoto's thyroiditis a clonal TcR-β gene rearrangement was found upon analysis of thyroid tissue samples, which were proven to contain significant T lymphocyte infiltrates (173).

Since RA, MS and Hashimoto's thyroiditis are not associated with the development of T cell malignancies in the local inflammatory infiltrates, the above described data suggest that the T lymphocyte infiltrates in autoimmune diseases represent antigen-restricted T cell responses. These T cell responses are not only antigenic-specific, but probably clonally-restricted as well (173).

DETECTION OF TWO OR MORE SUBCLONES WITHIN A MALIGNANCY

Malignant lymphoid cells are generally regarded as a (mono)clonal expansion of a single malignantly-transformed cell. However, subclones can develop during this expansion. In several lymphoid malignancies of B cell origin subclones have been found at diagnosis by use of Ig gene rearrangement studies and/or the application of anti-idiotype (Id) antibodies (Table 4). Multiple rearranged IgH gene bands have been found in precursor B-ALL (174-180), follicular lymphomas in immunocompetent individuals (181-187) and in immunodeficiency patients with EBV-associated lymphomas (85,92,93) (Table 4).

Multiple IgH gene rearrangements in precursor B-cell acute lymphoblastic leukemias

Several studies indicate that multiple rearranged IgH gene bands are detectable in 15 to 30% of all precursor B cell acute lymphoblastic leukemias (precursor B-ALL), as detected by

TABLE 4. Occurrence of subclones in B cell malignancies as detected by Ig gene analysis.

Type of B cell malignancy	Frequency of subclones	Possible causes of subclone formation	References
Precursor B-ALL	15-30%	- malignant transformation of an early precursor (B) cell with germline Ig genes, followed by Ig gene rearrangements in descendents of the malignant progenitor cell - secondary rearrangements CAUTION: hyperdiploidy 14 and other chromosome 14 aberrations should be excluded	173-180
Germinal-center-cell-derived lymphomas and immunocytomas	++[a]	- somatic mutations (occurring in both exons and introns of the expressed Ig gene loci) - isotype switch	181-187
EBV-associated lymphomas in immunodeficiency patients	++[b]	multiple lymphomas, caused by the same multistep oncogenic process	64,65,85, 92,93

a. The precise frequency of subclone formation in germinal-center-cell-derived lymphomas and immunocytomas has not yet been determined; probably more than half of them contain subclones with different idiotypes.
b. The frequency of multiple lymphomas in immunodeficiency patients is not known. This frequency will probably appear to be higher than expected when multiple biopsies from one patient are analyzed.

use of a JH probe in multiple restriction enzyme digests (174-180). The rearranged IgH gene bands in such ALL can vary in number from three to six or more and generally they differ in density (Figure 7). Glucose-6-phosphate dehydrogenase (G6PD) isotype analyses and cytogenetic analyses indicate that precursor B-ALL have a monoclonal origin (188-191). In only 5 out of 463 ALL patients (~1%) two cytogenetically independent cell populations have been observed (191). In only one out of these five cases more than two rearranged IgH bands were detected and additional studies on X-linked restriction fragment length polymorphism in three of these patients, suggested that the apparently-independent cell populations were derived from a common malignantly-transformed progenitor (191). Therefore, the presence of more than two rearranged IgH gene bands in multiple restriction enzyme digests can be attributed to the formation of subclones, based on the assumption that generally both IgH gene alleles are rearranged in precursor B-ALL cells (see Chapter 6.2). This is supported by the finding that the rearranged bands can often be paired according to their density (Figure 7).

However, one should be aware that cytogenetic aberrations in ALL often concern numerical aberrations. Hyperdiploidy (>46 chromosomes per cell) is frequently seen in ALL and may involve chromosome 14 (192,193), which contains the IgH gene locus (194,195). Also other cytogenetic aberrations (translocations, inversions, etc.) may involve the IgH gene locus on chromosome 14q32 (189,190). Therefore, cytogenetic data are necessary for discrimination between multiple rearranged bands due to the presence of extra chromosomes 14 or 14q32 aberrations on the one hand and multiple rearranged bands due to subclone formation on the other hand. This is illustrated by Figure 7, in which an ALL with three rearranged IgH gene bands was concluded to represent a monoclonal ALL rather than a biclonal ALL, because of

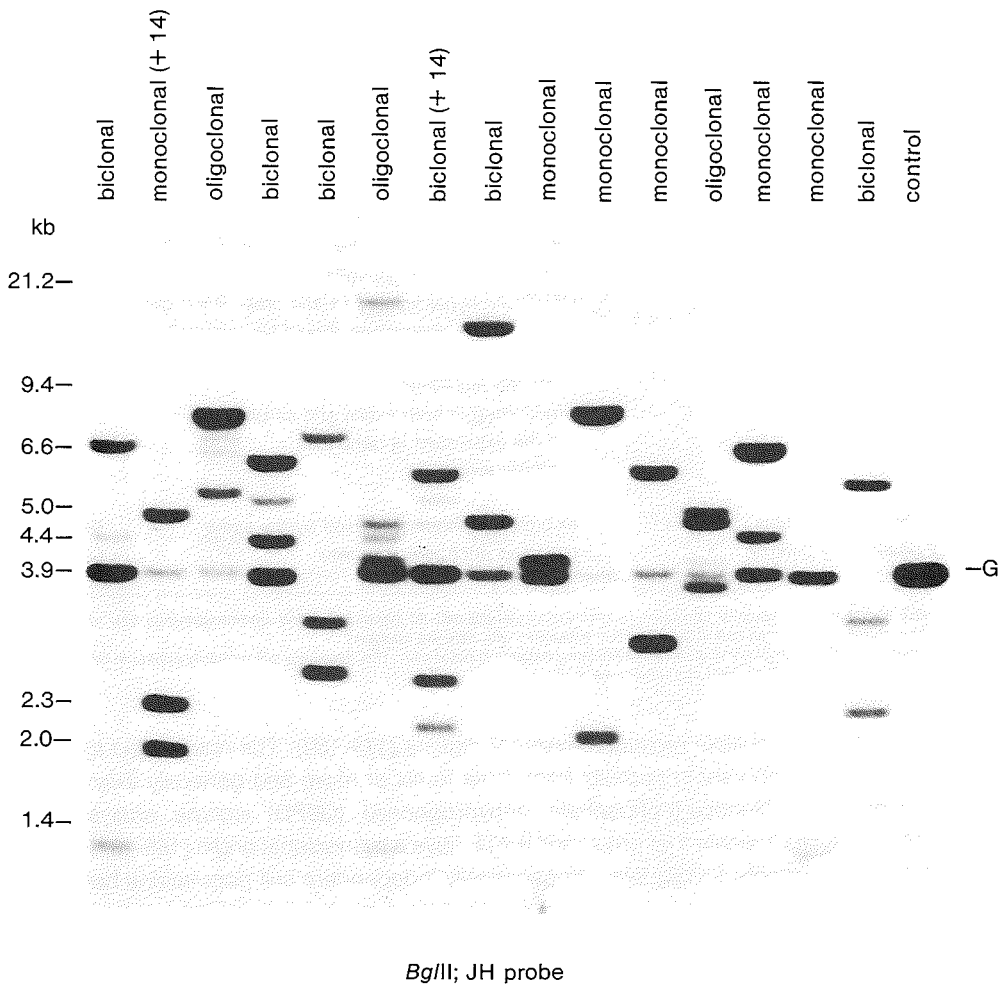


Figure 7. Analysis of the IgH genes in fifteen precursor B-ALL. Control DNA and DNA from precursor B-ALL cell samples were digested with *Bgl*II (and several other restriction enzymes; not shown), size separated and blotted to nylon membrane filters which were hybridized with the ^{32}P-labeled JH probe. In ten of the here presented precursor B-ALL more than two rearranged IgH gene bands were detected. In six cases three or four bands occurred, while in four other cases more than four bands were observed. The multiple rearranged IgH gene bands differed in density in most cases. In two patients (2nd and 7th lane) trisomy 14 (+14) was the cause of one of the extra bands. Based on the combined IgH gene and cytogenetic data it was concluded that six of the fifteen presented precursor B-ALL were monoclonal, another six were biclonal and three were oligoclonal (more than two clones).

the presence of three chromosomes 14 (trisomy 14; +14). In the same way, another ALL with trisomy 14 was concluded to be a biclonal ALL instead of an oligoclonal ALL (three clones or more) (Figure 7).

In one study a biclonal ALL (three rearranged IgH gene bands) and an oligoclonal ALL (seven rearranged IgH gene bands) were extensively analyzed by molecular cloning and

sequencing of junctional regions (178). It was demonstrated that each band represented different V-D-J or D-J rearrangements. These findings explain why the multiple rearranged IgH gene bands are generally found in all restriction enzyme digests. The sequence data revealed that the junctional regions of the IgH gene alleles were different and that the complementarity determining regions (CDR1 and CDR2) did not contain a single somatic mutation (178). Based on these data, the subclone formation in precursor B-ALL can be explained in two ways:

1. The oncogenic event occurs in a stem cell committed to the B cell lineage but lacking rearranged Ig genes. Subsequently IgH gene rearrangements occur in descendents of this malignantly-transformed cell. If the growth rates of these descendents are comparable, multiple rearranged bands will be detectable at diagnosis. However, if one of the descendents obtains a growth advantage during expansion of the ALL, only one or two rearranged bands will be detectable at diagnosis. Nevertheless in the latter case small subclones may be present, but too small to be detectable by use of the Southern blot technique (detection limit: 1 to 5%).
2. A second explanation for the subclone formation in precursor B-ALL is the occurrence of secondary rearrangements in cells of the malignantly-transformed precursor B cell clone. Secondary D to JH rearrangements can replace pre-existing D-JH rearrangements by joining an upstream D to a downstream JH gene segment (196). In addition, a novel VH gene segment can replace the VH segment of a pre-existing V-D-JH complex (197,198). These secondary rearrangements have been reported to occur in immature B cells (196-198). Also in case of secondary rearrangements the growth rates of the different clones determine the Ig gene rearrangement pattern at diagnosis.

Future studies will elucidate which of the here proposed mechanisms represents the cause of subclone formation in precursor B-ALL.

In their original report, Kitchingman and colleagues suggested that precursor B-ALL with multiple IgH gene rearrangements have a higher tendency to develop a relapse (175). This was not confirmed by other investigators (179,180). Our own preliminary results on 58 precursor B-ALL revealed a 46% higher relapse rate in the oligoclonal group (n=7) and a 9% higher relapse rate in the biclonal group (n=14) as compared to the monoclonal group (n=37), which had a relapse rate of 16% (median follow-up: 30 months) (Beishuizen et al., unpublished results). Therefore, our preliminary results suggest that the presence of subclones in precursor B-ALL represents a bad prognostic factor.

The occurrence of multiple rearranged TcR gene bands is rare. To our knowledge only one report describes an ALL with three rearranged TcR-β gene bands of different density (191). In our laboratory multiple rearranged TcR-β gene bands have been found in only 1 out of 30 T-ALL tested (Figure 8). Mechanisms for the development of subclones in T-ALL may be identical to those in precursor B-ALL, because secondary rearrangements have also been reported to occur in TcR genes (199,200).

Multiple subclones in germinal-center-cell-derived lymphomas

Using gene rearrangement studies and anti-Id antibodies it has been demonstrated that subclones can be present in some NHL at diagnosis (181,185,186). This especially concerns follicular lymphomas. First evidence for development of subclones in follicular lymphomas was

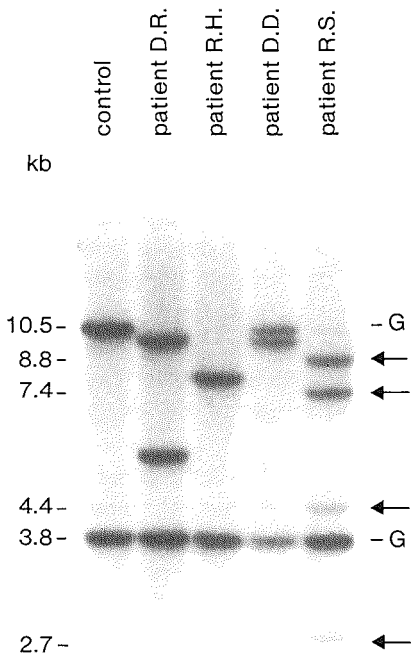


Figure 8. Analysis of the TcR-β genes in four T-ALL. Control DNA and DNA from the four T-ALL cell samples were digested with *Eco*RI, size separated and blotted to a nylon membrane filter which was hybridized with the Cβ probe. In patients DR, RH and DD one or two rearranged bands were observed, whereas in patient RS two strong and two weak rearranged bands were detectable. Cytogenetic analysis of the T-ALL cells from this patient did not reveal chromosome aberrations which could explain the presence of the two weak extra bands. Therefore, these findings indicate that the T-ALL of patient RS most probably consists of two subclones.

found in patients who were treated with anti-Id antibodies (182,183). In these patients tumor cell populations arose which were unreactive with the anti-Id antibodies, although Ig expression was retained (182,183). Cloning and sequence studies in one of these patients revealed that this was caused by subclone formation due to extensive somatic mutation in the functional VH gene region of the lymphoma cells (184). Additional sequence studies in a second patient revealed that numerous point mutations had occurred in the V region gene of the expressed IgH and IgL genes. The sequence data also indicated that the target for somatic mutation was much larger than the productive V gene region and extended into the J-C intron as well (186). It is obvious that this type of subclone formation proceeds with time and that tumor samples removed from the same follicular lymphoma patient, at different time points, will probably consist of different subclones (187).

The extensive somatic mutations in both exons and introns of the expressed Ig genes explain why this type of subclone formation is detectable by Southern blot analysis. The more extensive the somatic mutations, the more restriction sites will be affected. However, it is

unlikely that all restriction sites in and around the JH gene region are changed by somatic mutation. Therefore, extensive Southern blot analysis by use of multiple restriction enzyme digests will reveal that some restriction fragments differ between subclones, while others are identical (187).

In normal B cell development somatic mutations occur after antigenic stimulation during the immune response. The mutation mechanisms are not continuously active during immune responses, but especially occur during the generation of memory B cells (201,202). Most memory B cells switch to the expression of another isotype than IgM, which has been suggested to terminate the somatic mutation process (202). The generation of memory B cells most probably occurs in germinal centers, which are well-defined accumulations of proliferating B cells, antigen presenting cells and T cells (203,204). Follicular lymphomas and several other related lymphomas are considered to be derived from germinal center cells (187). This can explain why these lymphomas exhibit somatic-mutation-induced subclone formation. The above data indicate that isotype switch can occur in addition to somatic mutation (202). Isotype switch can change the size of restriction fragments in the JH gene region, depending on the restriction enzymes used (e.g. *Bam*HI and *Eco*RI). Detailed analysis of isotype switch can be performed by use of CH region probes (see Chapter 3.3). It should be noted that somatic mutations are probably restricted to the expressed IgH and IgL genes (202), while isotype switch frequently (~75% of cases) occurs on both the productive and non-productive alleles (205,206). This implies that changes in IgH gene rearrangement patterns can occur on both alleles. Therefore the use of multiple restriction enzyme digests is a prerequisite to prove the clonal origin of subclones in germinal-center-cell-derived lymphomas (187).

Multiple Ig gene rearrangements in EBV-associated lymphomas in immunodeficient patients

As outlined in one of the previous sections of this Chapter, the EBV-associated B cell lymphomas in immunodeficient patients are probably caused by a multistep oncogenic process, which is initiated by a polyclonal activation and proliferation of B lymphocytes (64,65). Due to impaired T cell regulation an uncontrolled proliferation of the B lymphocytes occurs in these patients, which may lead to oligoclonal or monoclonal outgrowth of B cells (64,65). If malignant transformation occurs in more than one clone, multiple lymphomas will arise. This has been observed in transplant patients, who had multiple lymphoma lesions with different Ig gene rearrangement patterns (93), as well as in AIDS patients, who had lesions with multiple rearranged IgH gene bands of different density (85,92).

ANALYSIS OF LYMPHOID MALIGNANCIES AT DIAGNOSIS AND SUBSEQUENT RELAPSES

Morphological techniques and immunologic marker analysis cannot give definite proof that the reoccurrence of a lymphoid malignancy indeed represents a relapse. A commen clonal origin can be proven by Southern blot analysis of Ig and TcR genes. Such information may be

important to choose the optimal treatment protocol. For instance, if a leukemia patient receives a BMT and subsequently a leukemia develops again, it is important to know whether this concerns a post-BMT relapse of the initial leukemia or whether this leukemia represents a new malignancy (207). In case of a new leukemia, the patient may benefit from the standard therapy protocols. However, if it concerns a relapse, the leukemic cells may have become resistant to the standard therapeutic regimens.

It is obvious that subclone formation as described in precursor B-ALL and follicular B cell lymphomas (175-187), will hamper the interpretation of the Southern blot data. This implies that (partly) identical Ig and TcR gene rearrangement patterns prove the common clonal origin of the malignant cells at diagnosis and relapse. If the rearrangement patterns are different, they should be interpreted with caution.

Proof that reoccurrence of a malignancy represents a relapse

In case of T cell malignancies no protein markers for clonality are available yet. Therefore TcR gene analysis is at present the only available technique to prove or exclude that reoccurrence of a T-NHL is indeed a relapse. Generally TcR-β gene analysis is sufficient for this purpose (Figure 9).

In some patients with B cell lymphoma the histomorphological characteristics of the lymphoma cells change over time from small cell size with follicular histomorphology to large cell size with diffuse histomorphology (208). Such changes often coincide with evolution of the disease, therapy resistance and short survival (208). By use of IgH and IgL gene analysis it can be demonstrated whether the morphological changes only represent phenotypic changes, while the Ig gene rearrangement pattern remains unchanged. Several examples of phenotypic shifts but genotypic identity in B-NHL have been published (187,208). On the other hand, changes in histomorphology may also be accompanied by changes in IgH and/or IgL gene rearrangements (187,208) (see below).

Also in precursor B-ALL and T-ALL as well as in acute myeloid leukemia (AML) with cross-lineage rearrangement of Ig or TcR genes, Southern blot analysis can be used for comparison of rearrangement patterns at diagnosis and relapse. Identical or partly identical patterns prove the clonal relationship between the leukemic cells. However, in case of different Ig or TcR gene rearrangement patterns, a common clonal origin of the leukemic cells cannot be excluded, because clonal evolution at the Ig or TcR gene level may have occurred (209-211).

Clonal evolution at first and/or subsequent relapses in ALL

From cytogenetic studies it is known that ALL cells at relapse often have additional chromosome aberrations as compared with the karyotype at initial diagnosis (212,213). Several reports indicate that also changes in Ig and TcR gene rearrangement patterns can be found in relapsing ALL (209-211).

In precursor B-ALL the changes often concern only one of the two rearranged IgH gene alleles (209-211) or additional rearrangements in the Igκ genes with identical IgH gene rear-

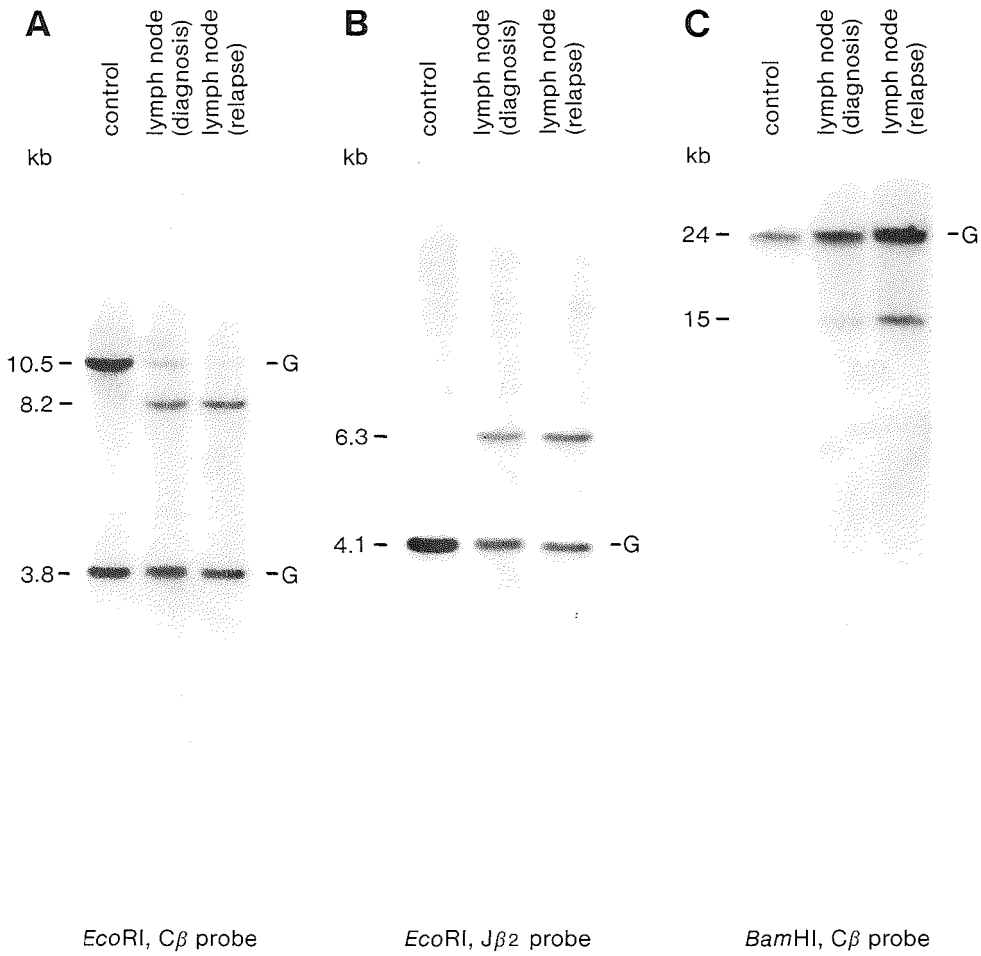


Figure 9. Analysis of the TcR-β genes of a T-NHL at diagnosis and at relapse. Control DNA and DNA from lymph node biopsies at diagnosis and relapse were digested with *Eco*RI (**A** and **B**) or *Bam*HI (**C**), size separated and blotted to nylon membrane filters, which were hybridized with the Cβ probe (**A** and **C**) or the Jβ2 probe (**B**). The patterns of rearranged TcR-β gene bands at diagnosis and relapse were identical.

rangements (210,211). To our experience these forms of clonal evolution at relapse occur in more than 50% of precursor B-ALL (Beishuizen et al., unpublished results). As can be anticipated, the changes in IgH gene rearrangement patterns are more extensive in oligoclonal precursor B-ALL, since in these leukemias a minor subclone may cause the relapse (Figure 10). Nevertheless, in most cases at least one rearranged Ig gene allele remains unchanged. If the IgH gene rearrangement patterns at diagnosis and relapse are completely different (with germline IgL and TcR genes), only cytogenetic analysis can prove the common clonal origin of the ALL cells.

Also in T-ALL changes in TcR gene rearrangements have been observed at relapse. Germ-

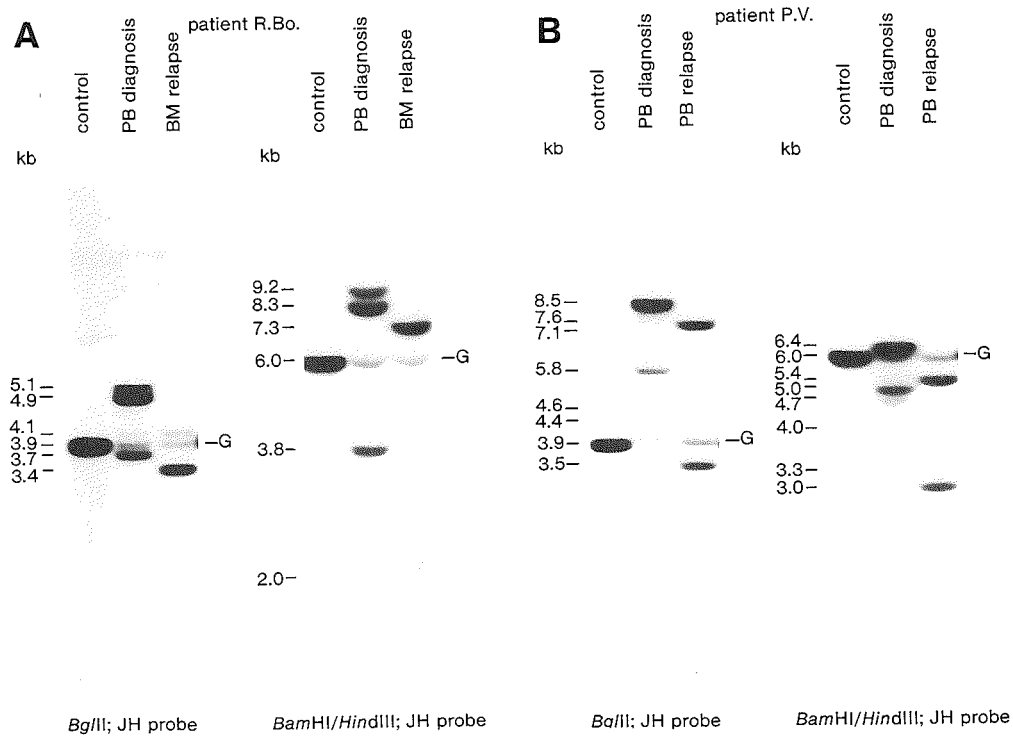


Figure 10. Analysis of the IgH genes at diagnosis and relapse in two patients with precursor B-ALL. Control DNA and DNA from PB and BM samples at diagnosis and relapse were digested with *Bgl*II or a combination of *Bam*HI and *Hin*dIII, size separated and blotted to nylon membrane filters which were hybridized with the ^{32}P-labeled JH probe. **A**: In patient RBo three distinct and one weak rearranged band were seen at diagnosis in both digests, whereas only one distinct and one weak rearranged band were detected at relapse. The weak band at diagnosis was probably identical to the weak band at relapse, but all other bands differed in size in both digests, indicating that clonal evolution had occurred in this precursor B-ALL at relapse. **B**: In patient PV several rearranged bands were seen at diagnosis in both digests: one distinct band, one weaker band and several (>4) faint bands. At relapse two rearranged bands of comparable density were seen in both digests, which probably have the same size as two of the faint bands at diagnosis. This is vaguely visible in the *Bgl*II filter. These data suggest that one of the small subclones present at diagnosis, has escaped from the cytotoxic treatment and caused the relapse.

line TcR-β genes at diagnosis can appear to be rearranged at relapse and vice versa (210). In a series of five T-ALL we have found a deletion of a rearranged TcR-δ gene in one T-ALL at relapse (Beishuizen et al., unpublished results). Our preliminary results suggest that the frequency of clonal evolution at the TcR gene level in T-ALL is lower than the frequency of clonal evolution at the Ig gene level in precursor B-ALL.

So far, no reports have been published about changes in cross-lineage TcR gene rearrangements in precursor B-ALL at relapse. Such information may be important, because TcR-γ and TcR-δ gene rearrangements occur in high frequency in precursor B-ALL (see Chapter 6.2) and therefore can serve as additional markers for clonality or common clonal origin.

Whether clonal evolution at the level of Ig and TcR gene rearrangements is related to short survival has not yet been investigated. Generally, subclone formation at relapse is related to therapy resistance and therefore is a poor prognostic factor.

Clonal evolution in B cell lymphomas

As described above, subclone formation due to somatic mutations occurs at high frequency in germinal-center-cell-derived lymphomas such as follicular lymphomas, but also in immunocytomas (181-187). In centrocytic lymphomas and CLL such somatic-mutation-induced subclone formation has not been observed (187). The somatic mutation process proceeds with time and may therefore result in multiple different subclones, of which at least a part will be detectable by use of Southern blot analysis of the IgH and IgL genes. The somatic mutations probably only occur on the expressed Ig gene alleles (202). The unexpressed IgH gene allele in most follicular lymphomas is involved in a t(14;18)(q32;q21) (214-217), in which the *bcl*-2 gene is juxtaposed to one of the JH gene segments (218-220). This *bcl*-2-JH rearrangement appears to be a stable marker, which is present in all subclones of a follicular lymphoma (186,187,221). Therefore the common clonal origin of subclones in follicular lymphomas can generally be proven by use of an appropriate *bcl*-2 probe (221).

PROOF OR EXCLUSION OF THE COMMON CLONAL ORIGIN OF TWO MALIGNANT LYMPHOID CELL POPULATIONS

In some patients with a B cell malignancy a second B cell malignancy may develop or sometimes two B cell malignancies are simultaneously detected in one patient. Southern blot analysis of IgH and IgL genes can indicate whether such malignancies have a common clonal origin (185-187,209,221-226), or whether they represent two independent B cell malignancies (227-232).

As indicated in the previous sections, the presence of two apparently-unrelated B cell malignancies should be discriminated from subclone formation due to somatic mutation (184-187,221). Therefore extensive IgH gene analysis with multiple restriction enzyme digests should be performed to prove that the IgH gene alleles in the two malignant cell populations are all rearranged differently (187). In two patients a lymphoma with bitypic IgL chain expression has been found. The $Ig\kappa^+$ and $Ig\lambda^+$ subclones of these two lymphomas appeared to be derived from a common progenitor, because they had at least one identical IgH gene rearrangement as well as one identical IgL gene rearrangement (187).

On the other hand, a patient has been described, in whom two lymph node biopsies, removed eighteen months apart, disclosed histologic and immunologic evidence for the presence of a B-NHL in the first lymph node and neoplastic lymphocytes of B and T cell origin in the second lymph node (233). Although the clonal B cell populations in both lymph nodes did not express IgH or IgL chains, the IgH, Igκ and Igλ gene rearrangements in the two lymph node biopsies were completely different. Therefore it was concluded that the patient had developed multiple lymphomas, of which the second B-NHL and the T-NHL may have been

induced by the cytostatic treatment of the first B-NHL (233).

Formally, Ig and TcR gene analyses cannot completely exclude the possibility that two different lymphoid malignancies have a common clonal origin, because the oncogenic event may have affected an immature lymphoid precursor cell before Ig or TcR gene rearrangement. Therefore, only cytogenetic analysis of both cell population can give definite proof concerning a possible common clonal origin.

One of the most intriguing clinical problems concerning clonal origin is the relationship between the B cell malignancies in Richter's syndrome.

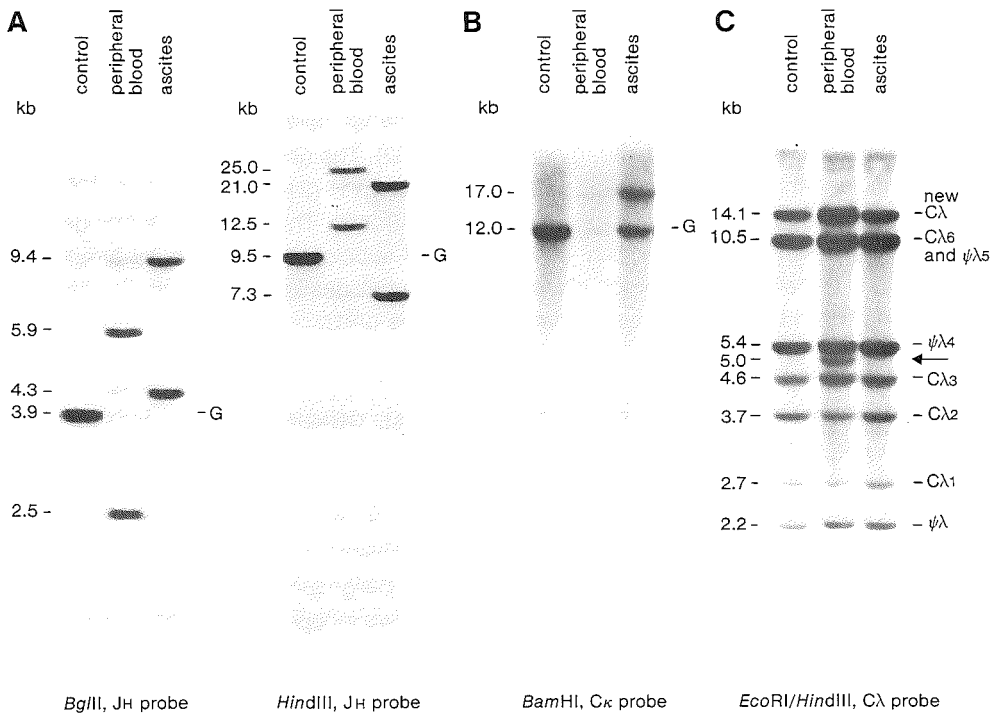


Figure 11. Analysis of the IgH, Igκ and Igλ genes of the B-CLL cells and B-NHL cells from patient LS with Richter's syndrome. Control DNA and DNA from PB cells ($Sm\lambda^+$ B-CLL cells) and ascites cells ($Sm\kappa^+$ B-NHL cells) were digested with *Bgl*II, *Hin*dIII, *Bam*HI or a combination of *Eco*RI and *Hin*dIII. The filters were hybridized with the JH probe, the Cκ probe or the Cλ probe. **A**: Analysis of the IgH genes revealed that the distinct rearranged bands of the PB cells and the ascites cells differed in both digests. Interestingly in the PB lanes of both filters two faint bands were observed, which corresponded to the distinct rearranged bands in the ascites lanes. This indicates that low numbers of B-NHL cells were also present in PB, which was confirmed by the immunophenotypic data (~5% of B-NHL cells in PB)(ref. 227). **B**: Analysis of the Igκ genes revealed that the Cκ gene segments were deleted in the PB cells on both alleles, whereas one rearranged and one germline Igκ gene band were found in the ascites cells. **C**: Analysis of the Igλ genes demonstrated that only in the PB cells one rearranged band was present (arrow) in addition to the seven germline bands. The Igλ genes of the ascites cells were in germline configuration. All data together indicate that the PB cells ($Sm\lambda^+$ B-CLL cells) had rearranged IgH genes, deleted Cκ gene segments and one rearranged Igλ gene allele, whereas the ascites cells ($Sm\kappa^+$ B-NHL cells) had rearranged IgH genes, one rearranged Igκ gene allele and germline Igλ genes. The completely different rearrangement patterns of the B-CLL cells and the B-NHL cells indicate that they represent two independent B cell malignancies.

Richter's syndrome

Richter's syndrome has been defined as the development of a large cell malignant lymphoma in a CLL patient, months to years after the initial diagnosis of the CLL (234,235). The relationship between both B cell malignancies in Richter's syndrome is difficult to establish on morphological criteria, because the NHL cells have an entirely different morphology then the CLL cells. In his original report Richter supposed the "reticular cell lymphoma" in a patient with CLL to be a second neoplasm (236). Based on the presence of identical IgH and IgL chain expression on the surface membrane of CLL and NHL cells in several patients with Richter's syndrome, it was concluded that Richter's syndrome in these cases represented a transformation or progression of a B-CLL clone to a B-NHL clone (237,238). However, the presence of identical IgH and IgL chains is not synonymous with clonality, since most B-CLL and B-NHL express the μ heavy chain and the statistical likelihood of two independent B cell clones to express the same IgL chain is more than 50% (227). In some reports indeed it has been demonstrated that both the CLL cells and NHL cells in Richter's syndrome may express μ heavy chains, but different IgL chains (227,228,239).

Southern blot analysis of IgH and IgL genes is a more accurate method to study the clonal origin of the two B cell malignancies in Richter's syndrome. During the last years we were able to study three cases of Richter's syndrome by Southern blot analysis.

In our first case of Richter's syndrome (patient LS) the CLL cells expressed Igμ,δ,λ, while the B-NHL cells were positive for Igμ,δ,κ. The difference in IgL chain expression already suggested that the CLL and NHL were independent malignancies. This was confirmed by extensive analysis of the IgH, Igκ and Igλ genes of both the PB cells (CLL cells) and the cells from the ascitic fluid (NHL cells): all IgH and IgL gene rearrangement patterns differed completely between the two cell populations (Figure 11). We therefore concluded that in this patient the B-NHL cannot be regarded as a progression of the B-CLL, but should be consid-

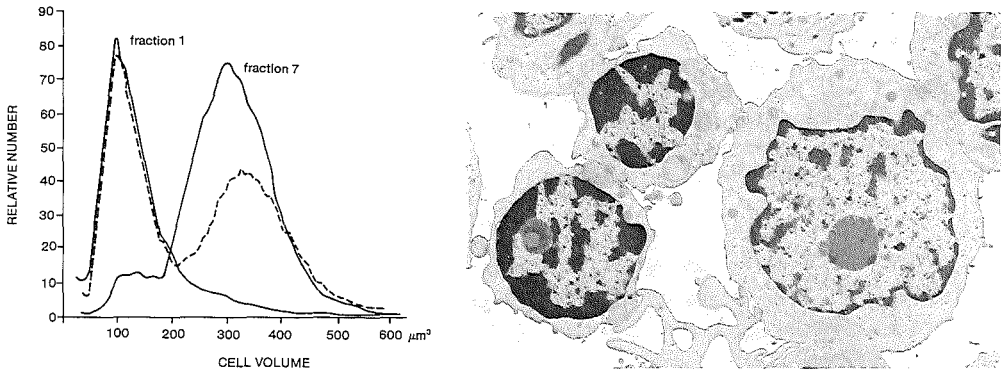


Figure 12. Separation of the small lymphocytes and the large lymphoid blasts in PB of patient AZ with Richter's syndrome (Michiels et al., ref. 226). *Left*: The small lymphocytes (~48% of the PB cells) and the large lymphoid blasts (~37% of PB cells) were separated according to their cell volume by centrifugal elutriation. Immunologic marker analysis of the cell fractions revealed that fraction 1 contained ~95% Smκ^+/CD5$^+$ B-CLL cells, while fraction 7 consisted of ~70% Smκ^+ B-NHL cells and ~25% CD14$^+$ monocytes. *Right*: Electron microscopic picture of the PB cells, showing two small lymphocytes and one large lymphoid blast cell.

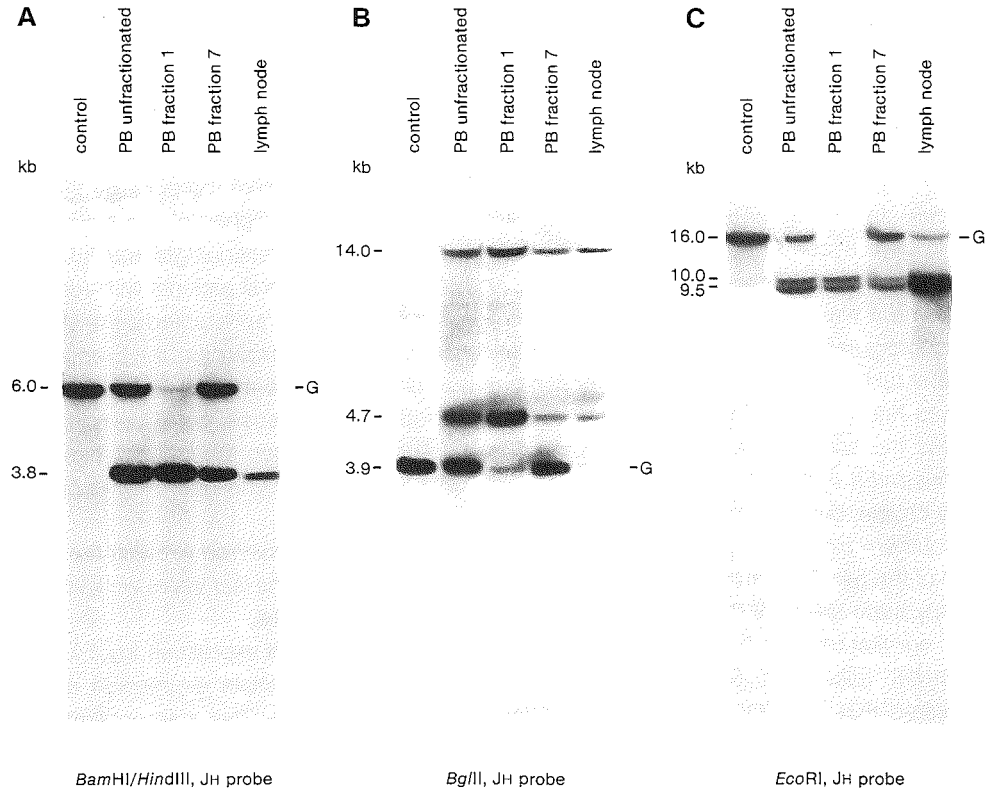


Figure 13. Analysis of the IgH genes of unfractionated PB cells, PB elutriation fraction 1 (Smκ^+ B-CLL cells), PB elutriation fraction 7 (Smκ^+ B-NHL cells) and lymph node cells (Smκ^+ B-NHL cells) from patient AZ with Richter's syndrome (Michiels et al., ref. 226). Control DNA and DNA from unfractionated PB cells, PB fractions 1 and 7 (see Figure 12) and lymph node cells were digested with a combination of *Bam*HI and *Hin*dIII (**A**), *Bgl*II (**B**) or *Eco*RI (**C**). The three filters were hybridized with the ^{32}P-labeled JH probe. The rearranged bands in the PB cells and lymph node cells were identical in all digests. In the *Bgl*II and *Eco*RI filters two rearranged bands occurred, while in the *Bam*HI/*Hin*dIII filter one rearranged band was observed which most probably consists of two co-migrating rearranged bands. The distinct germline bands in the unfractionated-PB-cell lane and in the PB-fraction-7 lane are probably due to the presence of non-malignant cells with germline IgH genes, such as T lymphocytes and/or monocytes. The combined data indicate that the B-CLL cells and the B-NHL cells have a common clonal origin.

ered as an independent B cell malignancy, which arose in a susceptible host (227).

The second case of Richter's syndrome (patient AZ) concerned a CLL patient who developed a centroblastic B-NHL with massive lymphadenopathy and involvement of PB. The differential count was: 37% large lymphoid blasts (B-NHL cells), 48% small lymphocytes (B-CLL cells), 9% granulocytes and 6% monocytes (Figure 12). The lymph node cells as well as both lymphoid cell populations in PB expressed Igμ,κ. For detailed analysis of the small lymphocytes and large lymphoid blasts in PB and optimal comparison with the lymph node cells, the PB cells were separated according to their cell volume by use of centrifugal elutriation (Figure 12). Fraction 1 contained the small PB cells (~95% CD5$^+$/Smμ,κ^+ B-CLL cells), while

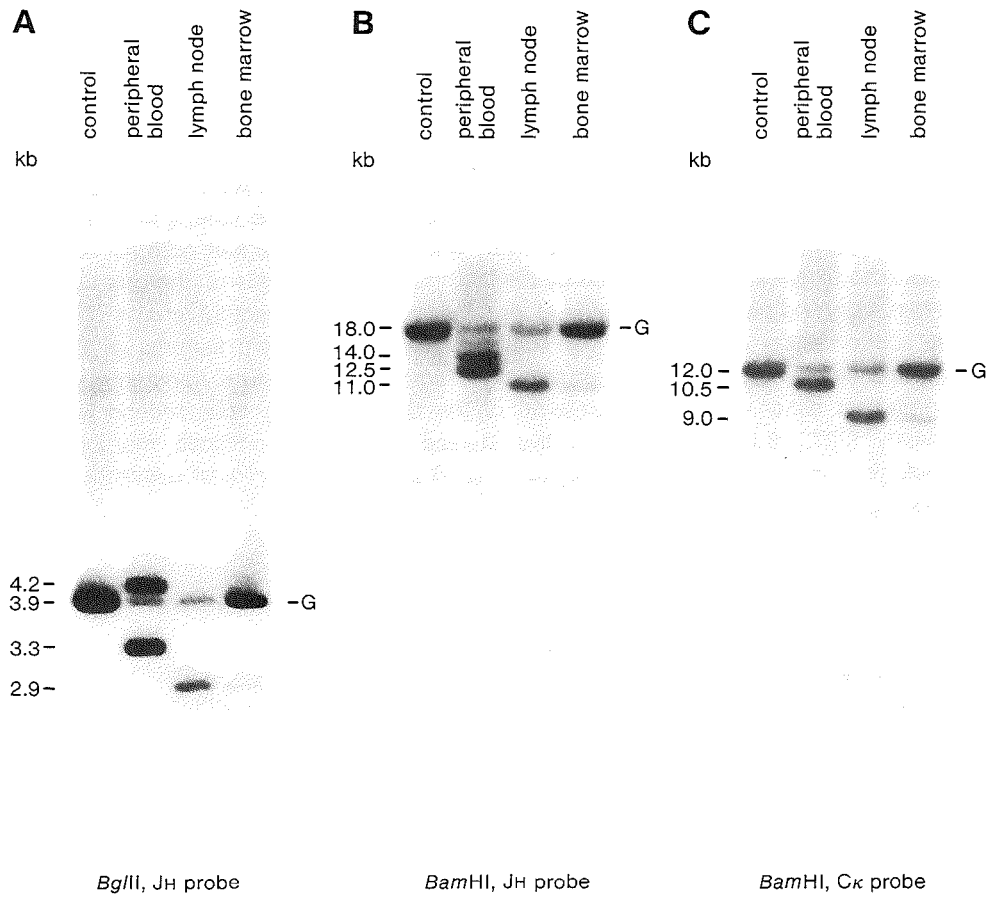


Figure 14. Analysis of the IgH and Igκ genes of PB cells, lymph node cells and BM cells from patient DJ with Richter's syndrome. Control DNA and DNA from PB cells (~60% Smκ$^+$ B-CLL cells), lymph node cells (Smκ$^+$ B-NHL cells) and BM cells (~10% Cyκ$^+$ plasma cells) were digested with *Bgl*II (**A**) or *Bam*HI (**B** and **C**). The filters were hybridized with the JH probe (**A** and **B**) or the Cκ probe (**C**). **A** and **B**: Analysis of the IgH genes revealed two germline bands in the PB lane, one rearranged band in the lymphnode lane and one weak rearranged band in the BM lane. The rearranged bands in the lymphnode lane and the BM lane were identical in both digests, but differed from the rearranged bands in the PB lane. **C**: Analysis of the Igκ genes demonstrated that the rearranged band in the lymphnode lane was identical to the weak rearranged band in the BM lane. The rearranged band in the PB lane had a different size. These data indicate that the Smκ$^+$ B-CLL cells in PB on the one hand and the Smκ$^+$ B-NHL cells in the lymph node and Cyκ$^+$ plasma cells in the BM on the other hand represent two independent malignancies. The B-NHL cells and the Cyκ$^+$ plasma cells in BM have a common clonal origin (see text).

fraction 7 contained the large PB cells (~70% Smμ,κ$^+$ large B-NHL cells and ~25% CD14$^+$ monocytes). Extensive Southern blot analysis of the IgH genes of both PB cell fractions as well as lymph node cells and unfractionated PB cells revealed that the B-CLL and B-NHL cells had completely identical IgH gene rearrangement patterns (Figure 13). This indicates that the NHL in this patient represented a clonal progression of the CLL cells (226).

In our third case of Richter's syndrome (patient DJ) the B-CLL was followed by an immunoblastic NHL and subsequent acute macroglobulinemia with hyper-viscosity syndrome. The B-CLL cells in PB, the B-NHL cells in the lymph nodes and the BM plasma cells expressed the same IgH and IgL chains: Igμ,κ. Southern blot analysis demonstrated that the IgH and Igκ gene rearrangements of the B-NHL cells and BM plasma cells were identical but differed from the B-CLL cells in PB (Figure 14). This indicates that the B-CLL and the immunoblastic NHL with subsequent evolution to acute macroglobulinemia represented two independent malignancies. A comparable case has been published by Giardina and colleagues (228). However, in their case the diagnosis was facilitated by the fact that the B-CLL cells on the one hand and the B-NHL cells and plasma cells on the other hand produced different IgL chains (228).

Our observations indicate that the immunologic and genotypic features of Richter's syndrome are more heterogeneous than assumed from the clinical picture. Apparently, the NHL may represent either a clonal progression of the CLL (222,226) or a second lymphoid malignancy (227,228,230,232). Prospective studies using the combined morphological, immunologic and Southern blot techniques should elucidate the clinical relevance of this heterogeneity in Richter's syndrome.

DETERMINATION OF THE DIFFERENTIATION LINEAGE OF A MALIGNANCY

In some cases it is not possible using immunologic marker analysis to determine whether the malignant cells belong to the B cell lineage or the T cell lineage, or whether it concerns a non-lymphoid malignancy.

It is generally assumed that Ig and TcR gene rearrangements are only functional in B and T cells, respectively. Nevertheless such rearrangements have been described to occur occasionally in cells from other lineages as well, such as Ig rearrangements in T-ALL and AML and TcR gene rearrangements in precursor B-ALL and AML (240,241; see Chapter 6.2 for details). Due to such cross-lineage rearrangements, the detection of Ig or TcR gene rearrangements in malignant cells does not provide definitive proof of the differentiation lineage to which the malignancy belongs. Despite this limitation in some lymphoproliferative diseases, Southern blot analysis of Ig and TcR genes can be of great value for lineage assignment of the malignant cells. Also the absence of Ig and TcR gene rearrangements can be informative.

Lennert's lymphoma

Lennert's lymphoma is an unusual form of malignant lymphoma which is characterized by the presence of a large number of epithelioid cells (242). Initially it was thought to represent a variant of Hodgkin's disease, but later it was concluded that the proliferating (Ki-67$^+$) CD4$^+$ T lymphocytes in Lennert's lymphoma probably are the malignant cells (242,243). The clonal origin of these CD4$^+$ T lymphocytes was proven by Southern blot analysis of the TcR genes. It appeared that in all studied cases of Lennert's lymphoma the TcR-β genes and TcR-γ genes were clonally rearranged and that in a part of them also clonally rearranged TcR-δ genes were present (244-246). The IgH genes were in germline configuration in all studied cases (244-246).

CD30$^+$ lymphomas and T-cell-like lymphomas

CD30 (Ki-1)$^+$ NHL are anaplastic large cell lymphomas, characterized by the proliferation of large bizarre cells, which show early involvement of the area around B cell follicles and tend to disseminate into the marginal sinuses (247). The expression of T cell and/or B cell markers in the absence of cytokeratin and/or macrophage markers suggests that the neoplastic cells are derived from activated (CD30$^+$) lymphoid cells. Most commonly they are of T cell origin and in some cases they represent cells of the B cell lineage (247,248).

Southern blot analysis has supported this assumption, because in most cases indeed clonally rearranged TcR genes have been found in concordance with the T cell phenotype of the CD30$^+$ neoplastic cells (246,248). In some other cases IgH gene rearrangements have been observed, generally in association with the expression of B cell antigens by the CD30$^+$ cells (246,248). In a few cases discordancies between marker expression and gene rearrangements have been found (248). This may be due to admixture of reactive cell populations, which is supported by the finding that the clonally rearranged TcR and/or Ig gene bands often are of low density (248). Also CD30$^+$ NHL with germline TcR and Ig genes have been reported (248; see also patient SM in Table 5 and Figure 15). These data indicate that most CD30$^+$ NHL probably are of T cell origin. However, Southern blot analysis cannot provide the desired information in each case.

TABLE 5. Immunologic marker analysis and Southern blot analysis of TcR and Ig genes in three malignant lymphomas with a few T cell characteristics.

	patient JW	patient SM	patient JS
Cell sample	peripheral blood	lymph node	skin biopsy
Immunophenotype[a]			
CD20 (B1)	0%	–	–
CD2 (Leu-5)	95%	±	+
CD3 (Leu-4)	3%	–	–
CD4 (Leu-3)	71%	–	–
CD5 (Leu-1)	79%	–	–
CD7 (Leu-9)	3%	–	+
CD8 (Leu-3)	3%	–	–
CD25 (IL-2R)	1%	+	NT
CD30 (Ki-1)	NT	+	NT
Southern blot analysis[b]			
TcR-β	R/R	G/G	G/G
TcR-γ	R/R	G/G	G/G
TcR-δ	D/D	G/G	G/G
IgH	G/G	G/G	G/G

a. Immunophenotypic data are derived from immunologic marker analysis of cells in suspension (positivity is given in percentages) or from immunologic marker analysis on frozen sections (expression indicated with symbols: –, only a few cells positive; ±, weak expression; +, majority of cells positive).

b. The Southern blot data are illustrated by Figure 5. The used letter codes indicate: G, germline; R, rearranged; D, deleted. The configuration of both alleles is given.

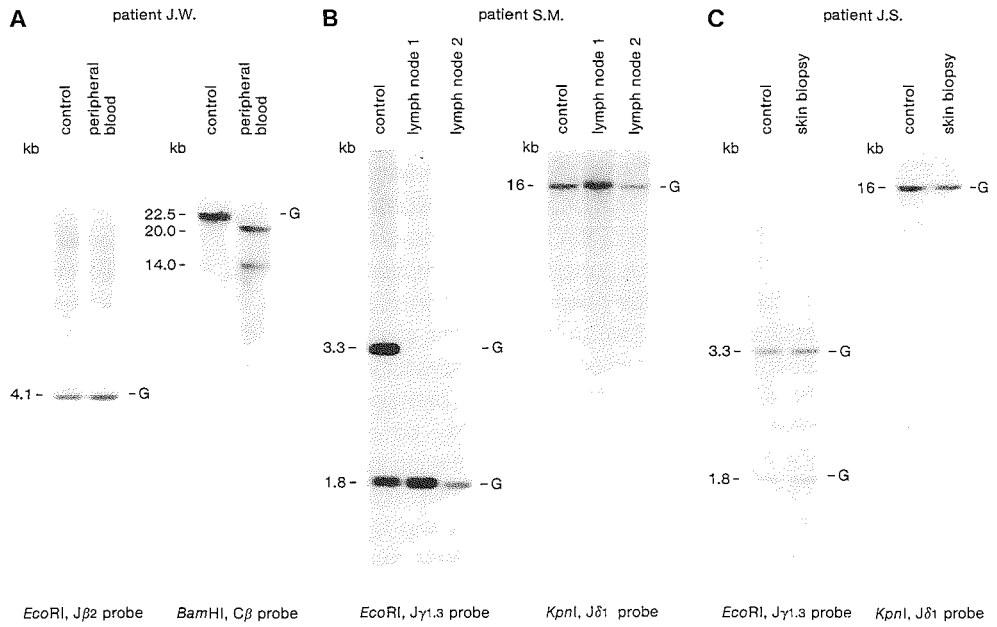


Figure 15. Analysis of the TcR-β, TcR-γ and TcR-δ genes in three malignant lymphomas with a few T cell characteristics (see Table 5). Control DNA and DNA from the PB cells of patient JW ($CD2^+/CD5^+$ T-NHL), two lymph node biopsies of patient SM ($CD2^{\pm}/CD30^+$ NHL) and a skin biopsy of patient JS ($CD2^+/CD7^+$ NHL) were digested with *Eco*RI, *Bam*HI or *Kpn*I. The filters were hybridized with the Cβ probe, Jβ2 probe, Jγ1.3 probe or Jδ1 probe (not all results are shown in the figure). **A**: Analysis of the TcR-β genes of the PB cells from patient JW revealed two rearranged bands in the *Bam*HI digest after hybridization with the Cβ probe, while no rearranged bands occurred in the *Eco*RI digest after hybridization with the Jβ2 probe. This indicates that both TcR-β gene alleles of the T-NHL cells in PB are probably rearranged to the β1 locus. **B**: Analysis of the TcR-γ and TcR-δ genes of the cells in both lymph node biopsies from patient SM revealed only germline bands. The absence of the 3.3-kb germline band in the *Eco*RI filter hybridized with the Jγ1.3 probe is due to a genetic polymorphism on both TcR-γ gene alleles (see Chapter 3.3). **C**: Analysis of the TcR-γ and TcR-δ genes of the cells in the skin biopsy of patient JS revealed only germline bands. Details concerning the malignant lymphomas of the three patients are summarized in Table 5.

The limitations of Southern blot analysis for lineage assignment are also illustrated by the finding that some T cell lymphomas or "T-cell-like" lymphomas have germline TcR genes (41; patient JS in Table 5 and Figure 15). Such lymphomas may have been derived from immature T cells before TcR gene rearrangements or from immature lymphoid cells which aberrantly exhibit some T cell characteristics.

Hodgkin's disease

Reed-Sternberg (RS) cells are pathognomonic of Hodgkin's disease and are considered to be the neoplastic cell population in this disease. The precise identity and origin of RS cells is still unclear. A large series of studies have tried to unravel this problem by analyzing the Ig

and TcR genes in biopsies from involved lymph nodes (245,246,249-259). Despite these efforts the data remained controversial. Some investigators have found rearranged Ig and/or TcR gene bands (generally of low density) (245,246,253-255), while in other studies such rearrangements were only sporadically observed (249-251,256-258). Also no clear positive correlation has been found between the absence or presence of Ig and/or TcR gene rearrangements on the one hand and percentages of RS cells on the other hand (250,252,258).

Therefore, we conclude that the Southern blot analyses have not resolved the RS cell enigma. Since Hodgkin's disease is associated with decreased T cell function, it might be that the occurrence of (small) clonal B and T cell populations is due to dysregulation of the immune response (260). This is supported by the finding that EBV-DNA has been detected in Hodgkin's disease biopsies with clonal Ig rearrangements (260).

Absence of Ig and TcR gene rearrangements

Gene rearrangements studies for lineage assignment have generally been used to support the B or T cell origin of a lymphoproliferative disease (10,11,15-17,41,245,246,248). However,

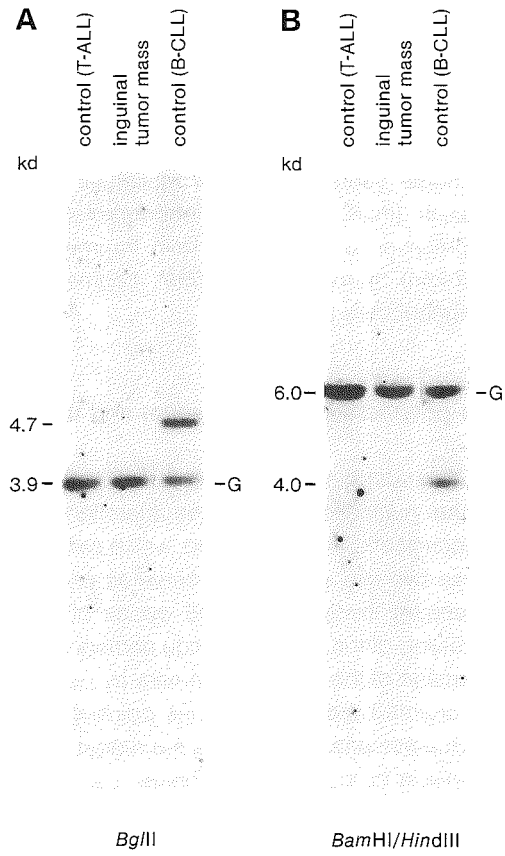


Figure 16. Analysis of the IgH genes of cells in an inguinal tumor mass which was thought to be an extramedullary plasmacytoma without Ig expression. Control DNA from a T-ALL and a B-CLL as well as DNA from the inguinal tumor mass were digested with *Bgl*II (**A**) or a combination of *Bam*HI and *Hin*dIII (**B**). Both filters were hybridized with the ^{32}P-labeled JH probe. Only germline bands occurred in the DNA from the inguinal tumor mass. Since plasma cells have rearranged IgH genes, it was concluded that the inguinal tumor mass did not represent an extramedullary plasmacytoma. Additional immunophenotyping revealed that it concerned an anaplastic carcinoma.

the absence of Ig and TcR gene rearrangements can be useful for lineage assignment in some cases, e.g. to support the myeloid origin of a malignancy (261).

We performed Southern blot analysis of an inguinal tumor mass, which was diagnosed to be an extramedullary plasmacytoma without Ig expression. Since all mature B cell malignancies have been reported to contain rearranged Ig genes (see Chapter 6.2), we analyzed the configuration of the IgH genes, which were found to be germline in all restriction enzyme digests (Figure 16). Subsequent additional immunophenotypic studies revealed that it concerned an anaplastic carcinoma.

DETECTION OF LOW NUMBERS OF MALIGNANT CELLS

The detection limit of routinely performed Southern blot analysis for the detection of clonally rearranged Ig and TcR genes is 1 to 5% (see Chapter 3.3). For many lymphoid malignancies such detection limits can also be reached by use of morphological techniques or immunologic marker analysis (19). Nevertheless, in some cases Southern blot analysis of Ig or TcR genes may be valuable for the detection of minimal disease (180,209).

After withdrawal of cytotoxic treatment in a patient with precursor B-ALL, the regenerating BM may be difficult to distinguish from a BM relapse, because many normal $TdT^+/CD10^+$ precursor B cells (10 to 20% and sometimes up to 50%) are present in regenerating BM samples (19). Southern blot analysis of the IgH genes of such regenerating BM samples in combination with immunophenotypic data will generally provide sufficient information for the correct diagnosis.

Some years ago a new molecular genetic technique, the so-called polymerase chain reaction (PCR), has been introduced (262-264). The PCR technique allows the selective amplification of a particular DNA fragment while it is still incorporated in total genomic DNA (262-264). If the involved DNA fragment is tumor-specific, detection of low numbers of malignant cells is possible.

The polymerase chain reaction and the detection of minimal residual disease

The specificity of the DNA amplification in the PCR technique depends on the availability of the nucleotide sequences that flank the region of interest. Therefore two synthetic oligonucleotides are prepared, which can hybridize to the flanking regions of opposite strands. These two oligonucleotides serve as primers for extension by a DNA polymerase. The PCR procedure involves repeated cycles of heat denaturation of the genomic DNA (~95°C), annealing of the oligonucleotide primers to their complementary sequences (55 to 60°C), and extension of the annealed primers by use of DNA polymerase (70 to 75°C). The two primers are oriented with their 3′ ends pointing towards each other, so that DNA synthesis by the polymerase proceeds across the region of interest, which is located between the primers. The extension products of one primer can serve as a template for the other primer. So each successive PCR cycle (denaturation, annealing and extension) essentially doubles the amount of DNA synthesized in the previous cycles. This results in an exponential amplification of the

DNA fragment of interest. The enzyme *Taq* polymerase is stable at the high denaturation temperature, which makes it unnecessary to add new polymerase after each cycle. This allows the PCR to continue for 20-30 cycles or more, theoretically resulting in 2^{20} to 2^{30} DNA fragments of interest (262-264), which can easily be detected in a dot blot or Southern blot by use of a ^{32}P-labeled probe, which specifically hybridizes to the amplified DNA fragment.

It is obvious that this PCR-mediated amplification of specific DNA fragments represents an excellent tool for the detection of low frequencies of tumor-specific DNA sequences (derived from low frequencies of tumor cells). Theoretically the detection limit is approximately 0.001 to 0.0001% (one malignant cell between 10^5 to 10^6 normal cells), based on the fact that a PCR tube can maximally contain 1 to 10 μg of DNA, while a human cell contains approximately 10 pg of DNA.

The PCR technique was first used for the detection of low numbers of malignant hematopoietic cells with a tumor-specific translocation, such as t(14;18)(q32;q21) or t(9;22)(q34;q11) (265-267). The two primers for these purposes were designed to recognize sequences at opposite sides of the breakpoint recombination area, so that the amplified DNA fragment represented the tumor-specific hybrid DNA segment.

Recent reports indicate that PCR-mediated amplification of the junctional regions of rearranged Ig and TcR genes can be used for the detection of minimal residual disease in lymphoid malignancies (268-270). This is based on the fact that the junctional region of rearranged Ig and TcR genes can vary enormously due to the joining of V, (D) and J gene segments and the insertion of nucleotides at the joining sites (see Chapter 3.2). The junctional regions probably differ in each lymphocyte (clone) and therefore also in each leukemia or lymphoma. It has been suggested that the PCR-mediated amplification of leukemia/lymphoma-specific junctional regions can be used in case of rearranged IgH genes (269), rearranged TcR-γ genes (268) and rearranged TcR-δ genes (270). However, it should be emphasized that oligoclonality at diagnosis and clonal evolution at relapse will severely hamper the application of this technique in precursor B-ALL and germinal-center-cell-derived lymphomas (see previous sections). This especially concerns subclone formation at the IgH gene level. Future studies will elucidate whether rearranged TcR-γ and TcR-δ are less prone to the development of additional changes. If so, then PCR-mediated amplification of TcR-γ and TcR-δ junctional regions will be an excellent procedure for the detection of minimal residual disease in precursor B-ALL with cross-lineage TcR gene rearrangements.

CONCLUSION

Southern blot analysis of Ig and TcR genes is a powerful method for the detection of clonal B and T cell proliferations. Although in the majority of patients with a lymphoproliferative disease morphological techniques and immunologic marker analysis give sufficient information, in some patients gene rearrangement studies are necessary to make the correct diagnosis. The application should be restricted to selected diagnostic purposes (see Table 1), since Southern blotting is relatively expensive and time-consuming. In addition, much background information about Ig and TcR gene analysis as well as broad experience are needed for correct interpretation of the Southern blot data. This is especially true for clinically-benign lym-

phoproliferations, which are frequently seen in immunodeficiency patients.

The application of Southern blot analysis of Ig and TcR genes for the detection of minimal residual disease is hampered by the relatively high detection limit (1 to 5%). On the other hand, the recently developed PCR technique for the selective amplification of leukemia/lymphoma-specific junctional regions seems to be promising for this purpose.

ACKNOWLEDGMENTS. We gratefully acknowledge Professor Dr. R. Benner, Dr. H. Hooijkaas and Dr. H.J. Adriaansen for their continuous support, Mr. T.M. van Os (Department of Immunology, Erasmus University, Rotterdam) and Mr. C. van Dijk (Interpoint, Hapert) for excellent assistance in the preparation of the figures and Mrs. J. de Goey-van Dooren, Ms. E.P. Schenkel and Ms. H.J. de Jager for their secretarial assistance.

Most of the figures and clinical cases in this Chapter concern patients of whom cell samples were sent to the Department of Immunology for diagnostic analyses. Therefore we wish to thank Professor Dr. J. Abels, Dr. Th.W. van den Akker, Dr. H.N.L.M. Bron, Dr. J. Burghouts, Dr. A. Dolman, Dr. K. van Groningen, Dr. H.L. Haak, Dr. A. Hagenbeek, Dr. K. Hählen, Dr. E.J. Harthoorn-Lasthuizen, Dr. H.P.T. van Helden, Dr. C. van der Heul, Dr. E.J. Hezik, Dr. A.C.J.M. Holdrinet, Professor Dr. Th. van Joost, Dr. M.C. Kappers-Klunne, Dr. Ph.M. Kluin, Dr. J.C. Kluin-Nelemans, Dr. E.W. Kuypers, Dr. Th. van der Kwast, Dr. H.C. Lequin, Dr. J.H.M. Lockefeer, Dr. J.J. Michiels, Dr. W. Molendijk, Professor Dr. H.J. Neijens, Dr. A. Rijlaarsdam, Dr. D. Roos, Dr. W. Sizoo, Dr. P. Sonneveld, Dr. M.B. van 't Veer, Dr. L.F. Verdonck, Professor Dr. J.M. Vossen, Dr. R. Weening, Dr. W. Weimar, Dr. E.R. van Wering, Dr. H. van de Willigen, Dr. G.E. van Zanen and Professor Dr. B.J.M. Zegers for sending patient cell samples.

REFERENCES

1. Siminovitch KA, Jensen JP, Epstein AL, Korsmeyer SJ. Immunoglobulin gene rearrangements and expression in diffuse histiocytic lymphomas reveal cellular lineage, molecular defects, and sites of chromosomal translocation. Blood 1986;67:391-7.
2. Picker LJ, Weiss LM, Medeiros LJ, Wood GS, Warnke RA. Immunophenotypic criteria for the diagnosis of non-Hodgkin's lymphoma. Am J Pathol 1987;128:181-201.
3. Schuurman H-J, Van Baarlen J, Huppes W, Lam BW, Verdonck LF, Van Unnik JAM. Immunophenotyping of non-Hodgkin's lymphoma: lack of correlation between immuno-phenotype and cell morphology. Am J Pathol 1987;129:140-51.
4. Aisenberg AC, Wilkes BM, Jacobson JO, Harris NL. Immunoglobulin gene rearrangements in adult non-Hodgkin's lymphoma. Am J Med 1987;82:738-44.
5. Sun T, Ngu M, Henshall J, Cuomo J, Eisenberg A, Benn P, Allen SL. Marker discrepancy as a diagnostic criterion for lymphoid neoplasms. Diagn Clin Immunol 1988;5:393-9.
6. Rudders RA, Dhillon S, Krontiris TG. Clonal analysis of untreated non-Hodgkin's lymphoma utilizing immunoglobulin gene rearrangement and immunophenotype. Cancer Res 1988;48:6272-7.
7. Henni T, Gaulard P, Divine M, Le Couedic J-P, Rocha D, Haioun C, Henni Z, Marolleau J-P, Pinaudeau Y, Goossens M, Farcet J-P, Reyes F. Comparison of genetic probe with immunophenotype analysis in lymphoproliferative disorders: a study of 87 cases. Blood 1988;6:1937-43.
8. De Jong D, Voetdijk BMH, Van Ommen GJB, Kluin-Nelemans JC, Beverstock GC, Kluin PM. Translocation t(14;18) in B cell lymphomas as a cause for defective immunoglobulin production. J Exp Med 1989;169:613-24.
9. Williams ME, Innes DJ, Borowitz MJ, Lovell MA, Swerdlow SH, Hurtubise PE, Brynes RK, Chan WC, Byrne GE Jr, Whitcomb CC, Thomas IV CY. Immunoglobulin and T cell receptor gene rearrangements in human lymphoma and leukemia. Blood 1987;69:79-86.
10. Borgonovo Brandter L, Smith CIE, Hammarström L, Lindemalm C, Christensson B. Clonal immunoglobulin gene rearrangements in primary mediastinal clear cell lymphomas. Leukemia 1989;3:122-9.
11. Cleary ML, Trela MJ, Weiss LM, Warnke R, Sklar J. Most null large cell lymphomas are B lineage neoplasms.

Lab Invest 1985;53:521-5.
12. Arnold A, Cossman J, Bakhshi A, Jaffe ES, Waldmann TA, Korsmeyer SJ. Immunoglobulin-gene rearrangements as unique clonal markers in human lymphoid neoplasms. N Engl J Med 1983;309:1593-9.
13. Jaffe ES, Longo DL, Cossman J, Hsu S-M, Arnold A, Korsmeyer SJ. Diffuse B cell lymphomas with T cell predominance in patients with follicular lymphoma or "pseudo T cell lymphoma". Lab Invest 1984;50:27A-8A.
14. Jaffe ES. Pathologic and clinical spectrum of post-thymic T-cell malignancies. Cancer Invest 1984;2:413-26.
15. Friedlander M, Zalcberg J, Patterson B, Minden M, Bergsagel DE. Apparent T-cell neoplasms with immunoglobulin gene rearrangements. Lancet 1985;ii:670.
16. Ramsay AD, Smith WJ, Isaacson PG. T-cell-rich B-cell lymphoma. Am J Surg Pathol 1988;12:433-43.
17. Scorpa A, Bonetti F, Zamboni G, Menestrina F, Chilosi M. T-cell-rich B-cell lymphoma. Am J Surg Pathol 1989;13:335-7.
18. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
19. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: M Nijhoff Publishers, 1986:113-33.
20. Van Dongen JJM, Hooijkaas H, Comans-Bitter M, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.
21. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res 1981;5:301-9.
22. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: M Nijhoff Publishers, 1984:67-81.
23. Weiss LM, Crabtree GS, Rousse RV, Warnke RA. Morphologic and immunologic characterization of 50 peripheral T-cell lymphomas. Am J Pathol 1985;118:316-24.
24. Flug F, Pelicci P-G, Bonetti F, Knowles II DM, Dalla-Favera R. T-cell receptor gene rearrangements as markers of lineage and clonality in T-cell neoplasms. Proc Natl Acad Sci USA 1985;82:3460-4.
25. Waldmann TA, Davis MM, Bongiovanni KF, Korsmeyer SJ. Rearrangements of genes for the antigen receptor on T cells as markers of lineage and clonality in human lymphoid neoplasms. N Engl J Med 1985;313:776-83.
26. O'Connor NTJ, Weatherall DJ, Feller AC, Jones D, Pallesen G, Stein H, Wainscoat JS, Gatter KC, Isaacson P, Lennert K, Ramsey A, Wright DH, Mason DY. Rearrangement of the T-cell-receptor β-chain gene in the diagnosis of lymphoproliferative disorders. Lancet 1985;i:1295-7.
27. Bertness V, Kirsch I, Hollis G, Johnson B, Bunn PA. T-cell receptor gene rearrangements as clinical markers of human T-cell lymphomas. N Engl J Med 1985;313:534-8.
28. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germ-line variable genes belonging to two subgroups. Cell 1986;45:237-46.
29. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
30. Forster A, Huck S, Ghanem N, Lefranc M-P, Rabbitts TH. New subgroups in the human T cell rearranging Vγ gene locus. EMBO J 1987;6:1945-50.
31. Chen Z, Font MP, Loiseau P, Bories JC, Degos L, Lefranc MP, Sigaux F. The human T-cell Vγ gene locus: cloning of new segments and study of Vγ rearrangements in neoplastic T and B cells. Blood 1988;72:776-83.
32. Satyanarayana K, Hata S, Devlin P, Roncarolo MG, De Vries JE, Spits H, Strominger JL, Krangel MS. Genomic organization of the human T-cell antigen-receptor α/δ locus. Proc Natl Acad Sci USA 1988;85:8166-70.
33. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. The human T-cell receptor delta chain: genomic

organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
34. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
35. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
36. Lanier LL, Ruitenberg J, Bolhuis RLH, Borst J, Phillips JH, Testi R. Structural and serological heterogeneity of γ/δ T cell antigen receptor expression in thymus and peripheral blood. Eur J Immunol 1988;18:1985-92.
37. Triebel F, Hercend T. Subpopulations of human peripheral T gamma delta lymphocytes. Immunol Today 1989;10:186-8.
38. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.
39. Coupe M, Foroni L, Stamp G, Lovatt D, Barnard M, Krausz T, Kalofonos H, Epenetos A, Luzzatto L, Cox TM. Clonal rearrangement of the T-cell receptor γ gene associated with a bizarre lymphoproliferative syndrome. Eur J Haematol 1988;41:289-94.
40. Matthews JH, Smith NA, Foroni L. A case of angioimmunoblastic lymphadenopathy associated with a long spontaneous remission, retrobulbar neuritis, a clonal rearrangement of the T-cell receptor gamma chain gene and an unusual marrow infiltration. Eur J Haematol 1988;41:295-301.
41. Weiss LM, Picker LJ, Grogan TM, Warnke RA, Sklar J. Absence of clonal beta and gamma T-cell receptor gene rearrangements in a subset of peripheral T-cell lymphomas. Am J Pathol 1988;130:436-42.
42. Smith JL, Haegert DG, Hodges E, Stacey GN, Howell WM, Wright DH, Jones DB. Phenotypic and genotypic heterogeneity of peripheral T-cell lymphoma. Br J Cancer 1988;58:723-9.
43. LeBoit PE, Parslow TG. Gene rearrangements in lymphoma: applications to dermatopathology. Am J Dermatopathol 1987;9:212-8.
44. Slater DN. Recent developments in cutaneous lymphoproliferative disorders. J Pathol 1987;153:5-19.
45. Lutzner M, Edelson R, Schein P, Green I, Kirkpatrick C, Ahmed A. Cutaneous T-cell lymphomas: the Sézary syndrome, mycosis fungoides, and related disorders. Ann Intern Med 1975;83:534-52.
46. Weiss LM, Hu E, Wood GS, Moulds G, Cleary ML, Warnke R, Sklar J. Clonal rearrangements of T-cell receptor genes in mycosis fungoides and dermatopathic lymphadenopathy. N Engl J Med 1985;313:539-44.
47. Ralfkiaer E, O'Connor NTJ, Crick J, Wantzin GL, Mason DY. Genotypic analysis of cutaneous T-cell lymphomas. J Invest Dermatol 1987;88:762-5.
48. Fuks ZY, Bagshaw MA, Farber EM. Prognostic signs and the management of the mycosis fungoides. Cancer 1973;32:1385-95.
49. Colby TV, Burke JS, Hoppe RT. Lymph node biopsy in mycosis fungoides. Cancer 1981;47:351-9.
50. Weiss LM, Wood GS, Hu E, Abel EA, Hoppe RT, Sklar J. Detection of clonal T-cell receptor gene rearrangements in the peripheral blood of patients with mycosis fungoides/Sezary syndrome. J Invest Dermatol 1989;92:601-4.
51. Vonderheid EC, Sobal EL, Nowell PC, Finan JB, Helfrich MK, Whipple DS. Diagnostic and prognostic significance of Sézary cells in peripheral blood smears from patients with cutaneous T cell lymphoma. Blood 1985;66:358-66.
52. Lamberg SI, Green SB, Byar DP, Block JB, Clendenning WE, Douglass MC, Epstein EH Jr, Fuks ZY, Golitz LE, Lorincz AL, McBurney EI, Michel B, Roenigk HH Jr, Van Scott EJ, Vonderheid EC. Clinical staging for cutaneous T-cell lymphoma. Ann Intern Med 1984;100:187-92.
53. Uchiyama T, Yodoi J, Sagawa K, Takatsuki K, Uchino H. Adult T-cell leukemia: clinical and hematologic features of 16 cases. Blood 1977;50:481-92.
54. Catovsky D, Rose M, Goolden AWG, White JM, Bourikas G, Brownell AI, Blattner WA, Greaves MF, Galton DAG, McCluskey DR, Lampert I, Ireland R, Bridges JM, Gallo RC. Adult T-cell lymphoma-leukaemia in blacks from the West Indies. Lancet 1982;i:639-43.
55. Matsuoka M, Hagiya M, Hattori T, Asou N, Mueda S, Shimada K, Tsai SC, Sakano H, Takatsuki K. Gene rearrangements of T cell receptor β and γ chains in HTLV-I infected primary neoplastic T cells. Leukemia 1988;2:84-90.
56. Weiss LM, Wood GS, Trela M, Warnke RA, Sklar J. Clonal T-cell populations in lymphomatoid papulosis:

evidence of a lymphoproliferative origin for a clinically benign disease. N Engl J Med 1986;315:475-9.
57. Kadin ME, Vonderheid EC, Sako D, Clayton LK, Olbricht S. Clonal composition of T cells in lymphomatoid papulosis. Am J Pathol 1987;126:13-7.
58. Weiss LM, Wood GS, Ellisen LW, Reynolds TC, Sklar J. Clonal T-cell populations in pityriasis lichenoides et varioliformis acuta (Mucha-Habermann disease). Am J Pathol 1987;126:417-21.
59. LeBoit PE, Beckstead JH, Bond B, Epstein WL, Frieden IJ, Parslow TG. Granulomatous slack skin: clonal rearrangement of the T-cell receptor β gene is evidence for the lymphoproliferative nature of a cutaneous elastolytic disorder. J Invest Dermatol 1987;89:183-6.
60. Wood GS, Weiss LM, Hu C-H, Abel EA, Hoppe RT, Warnke RA, Sklar J. T-cell antigen deficiencies and clonal rearrangement of T-cell receptor genes in pagetoid reticulosis (woringer Kolopp disease). N Eng J Med 1988;318:164-7.
61. Radl J. Monoclonal gammapathies: an attempt at a new classification. Neth J Med 1985;28:134-7.
62. Oshimi K. Granular lymphocyte proliferative disorders: report of 12 cases and review of the literature. Leukemia 1988;2:617-27.
63. Gerritsen E, Vossen J, Van Tol M, Jol-Van der Zijde C, Van der Weijden-Ragas R, Radl J. Monoclonal gammopathies in children. J Clin Immunol 1989;9:296-305.
64. Hanto DW, Frizzera G, Gajl-Peczalska KJ, Simmons RL. Epstein-Barr virus, immunodeficiency, and B cell lymphoproliferation. Transplantation 1985;39:461-72.
65. List AF, Greco FA, Vogler LB. Lymphoproliferative diseases in immunocompromised hosts: the role of Epstein-Barr virus. J Clin Oncol 1987;5:1673-89.
66. Frizzera G, Kaneko Y, Sakurai M. Angioimmunoblastic lymphadenopathy and related disorders: a retrospective look in search of definitions. Leukemia 1989;3:1-5.
67. Kyle RA, Lust JA. Monoclonal gammopathies of undetermined significance. Semin Hematol 1989;26:176-200.
68. Hammarström L, Smith CIE. Frequent occurrence of monoclonal gammopathies with an imbalanced light-chain ratio following bone marrow transplantation. Transplantation 1987;43:447-9.
69. Radl J, Valentijn RM, Haaijman JJ, Paul LC. Monoclonal gammapathies in patients undergoing immunosuppressive treatment after renal transplantation. Clin Immunol Immunopathol 1985;37:98-102.
70. Radl J, Weis J, Hoogeveen CM. Immunoblotting with (sub)class-specific antibodies reveals a high frequency of monoclonal gammopathies in persons thought to be immunodeficient. Clin Chem 1988;34:1839-42.
71. Van den Akker TW, Gillen AP, Bril H, Benner R, Radl J. Increased incidence of transient homogeneous immunoglobulins in irradiated and reconstituted C57BL/KaLwRij mice treated with 2´-deoxyguanosine. Clin Exp Immunol 1983;54:411-7.
72. Van den Akker TW, Tio-Gillen AP, Solleveld HA, Benner R, Radl J. The influence of T cells on homogeneous immunoglobulins in sera of athymic nude mice during aging. Scand J Immunol 1988;28:359-65.
73. Crawford J, Eye MK, Cohen HJ. Evaluation of monoclonal gammopathies in the "well" elderly. Am J Med 1987;82:39-45.
74. Kyle RA. "Benign" monoclonal gammopathy: a misnomer? JAMA 1984;251:1849-54.
75. Reece ER, Gartner JG, Seemayer TA, Joncas JH, Pagano JS. Epstein-Barr virus in a malignant lymphoproliferative disorder of B-cells occurring after thymic epithelial transplantation for combined immunodeficiency. Cancer Res 1981;41:4243-7.
76. Shearer WT, Ritz J, Finegold MJ, Guerra IC, Rosenblatt HM, Lewis DE, Pollack MS, Taber LH, Sumaya CV, Grumet FC, Cleary ML, Warnke R, Sklar J. Epstein-Barr virus-associated B-cell proliferations of diverse clonal origins after bone marrow transplantation in a 12-year-old patient with severe combined immunodeficiency. N Engl J Med 1985;312:1151-9.
77. Hanto DW, Frizzera G, Purtilo DT, Sakamoto K, Sullivan JL, Saemundsen AK, Klein G, Simmons RL, Najarian JS. Clinical spectrum of lymphoproliferative disorders in renal transplant recipients and evidence for the role of Epstein-Barr virus. Cancer Res 1981;41:4253-61.
78. Saemundsen AK, Klein G, Cleary M, Warnke R. Epstein-Barr-virus-carrying lymphoma in cardiac transplant recipient. Lancet 1982;ii:158.
79. Starzl TE, Porter KA, Iwatsuki S, Rosenthal JT, Shaw BW Jr, Atchison RW, Nalesnik MA, Ho M, Griffith BP, Hakala TR, Hardesty RL, Jaffe R, Bahnson HT. Reversibility of lymphomas and lymphoproliferative lesions

developing under cyclosporin-steroid therapy. Lancet 1984;i:583-7.
80. Ho M, Miller G, Atchison RW, Breinig MK, Dummer JS, Andiman W, Starzl TE, Eastman R, Griffith BP, Hardesty RL, Bahnson HT, Hakala TR, Rosenthal JT. Epstein-Barr virus infections and DNA hybridization studies in posttransplantation lymphoma and lymphoproliferative lesions: the role of primary infection. J Infect Dis 1985;152:876-86.
81. Forman SJ, Sullivan JL, Wright C, Ratech H, Racklin B, Blume KG. Epstein-Barr-virus related malignant B cell lymphoplasmacytic lymphoma following allogeneic bone marrow transplantation for aplastic anemia. Transplantation 1987;44:244-9.
82. Zutter MM, Martin PJ, Sale GE, Shulman HM, Fisher L, Thomes ED, Durnam DM. Epstein-Barr virus lymphoproliferation after bone marrow transplantation. Blood 1988;72:520-9.
83. Davey DD, Kamat D, Laszewski M, Goeken JA, Kemp JD, Trigg ME, Purtilo DT, Davis J, Dick FR. Epstein-Barr virus-related lymphoproliferative disorders following bone marrow transplantation: an immunologic and genotypic analysis. Modern Pathol 1989;2:27-34.
84. Birx DL, Redfield RR, Tosato G. Defective regulation of Epstein-Barr virus infection in patients with acquired immunodeficiency syndrome (AIDS) or AIDS-related disorders. N Engl J Med 1986;314:874-9.
85. Pelicci P-G, Knowles II DM, Arlin ZA, Wieczorek R, Luciw P, Dina D, Basilico C, Dalla-Favera R. Multiple monoclonal B cell expansions and c-*myc* oncogene rearrrangements in acquired immune deficiency syndrome-related lymphoproliferative disorders. J Exp Med 1986;164:2049-76.
86. Spector BD, Perry III GS, Kersey JH. Genetically determined immunodeficiency diseases (GDID) and malignancy: report from the immunodeficiency-cancer registry. Clin Immunol Immunopathol 1978;11:12-29.
87. Frizzera G, Rosai J, Dehner LP, Spector BD, Kersey JH. Lymphoreticular disorders in primary immunodeficiencies: new findings based on an up-to-date histologic classification of 35 cases. Cancer 1980;46:692-9.
88. Hanto DW, Gajl-Peczalska KJ, Frizzera G, Arthur DC, Balfour HH, McClain K, Simmons RL, Najarian JS. Epstein-Barr virus (EBV) induced polyclonal and monoclonal B-cell lymphoproliferative diseases occurring after renal transpantation: clinical, pathologic, and virologic findings and implications for therapy. Ann Surg 1983;198:356-69.
89. Cleary ML, Warnke R, Sklar J. Monoclonality of lymphoproliferative lesions in cardiac-transplant recipients. N Engl J Med 1984;310:477-82.
90. Frizzera G, Hanto DW, Gajl-Peczalska KJ, Rosai J, McKenna RW, Sibley RK, Holahan KP, Lindquist LL. Polymorphic diffuse B-cell hyperplasias and lymphomas in renal transplant recipients. Cancer Res 1981;41:4262-79.
91. Hanto DW, Frizzera G, Gajl-Peczalska KJ, Sakamoto K, Purtilo DT, Balfour HH Jr, Simmons RL, Najarian JS. Epstein-Barr virus-induced B-cell lymphoma after renal transplantation: acyclovir therapy and transition from polyclonal of monoclonal B-cell proliferation. N Engl J Med 1982;306:913-8.
92. Knowles DM, Chamulak GA, Subar M, Burke JS, Dugan M, Wernz J, Slywotzky C, Pelicci P-G, Dalla-Favera R, Raphael B. Lymphoid neoplasia associated with the acquired immunodeficiency syndrome (AIDS): the New York University Medical Center experience with 105 patients (1981-1986). Ann Intern Med 1988;108:744-53.
93. Cleary ML, Sklar J. Lymphoproliferative disorders in cardiac transplant recipients are multiclonal lymphomas. Lancet 1984;ii:489-93.
94. Klein G, Klein E. Evolution of tumours and the impact of molecular oncology. Nature 1985;315:190-5.
95. Frizzera G. Castleman's disease: more questions than answers. Human Pathol 1985;16:202-5.
96. Hanson CA, Frizzera G, Patton DF, Peterson BA, McClain KL, Gajl-Peczalska KJ, Kersey JH. Clonal rearrangement for immunoglobulin and T-cell receptor genes in systemic Castleman's disease: association with Epstein-Barr virus. Am J Pathol 1988;131:84-91.
97. Knowles DM, Athan E, Ubriaco A, McNally L, Inghirami G, Wieczorek R, Finfer M, Jakobiec FA. Extranodal noncutaneous lymphoid hyperplasias represent a continuous spectrum of B-cell neoplasia: demonstration by molecular genetic analyses. Blood 1989;73:1635-45.
98. Knowles II DM, Jakobiec FA. Cell marker analysis of extranodal lymphoid infiltrates: to what extent does the determination of mono- or polyclonality resolve the diagnostic dilemma of malignant lymphoma *v* pseudolymphoma in an extranodal site? Semin Diagn Pathol 1985;2:163-8.
99. Brooks JJ, Enterline HT. Gastric pseudolymphoma: its three subtypes and relation to lymphoma. Cancer

1983;51:476-86.
100. Koss MN, Hochholzer L, Nichols PW, Wehunt WD, Lazarus AA. Primary non-Hodgkin's lymphoma and pseudolymphoma of lung: a study of 161 patients. Hum Pathol 1983;14:1024-38.
101. Kennedy JL, Nathwani BN, Burke JS, Hill LR, Rappaport H. Pulmonary lymphomas and other pulmonary lymphoid lesions: a clinicopathologic and immunologic study of 64 patients. Cancer 1985;56:539-52.
102. Anderson LG, Talal N. The spectrum of benign to malignant lymphoproliferation in Sjögren's syndrome. Clin Exp Immunol 1971;9:199-221.
103. Holm L-E, Blomgren H, Löwhagen T. Cancer risks in patients with chronic lymphocytic thyroiditis. N Engl J Med 1985;312:601-4.
104. Kato I, Tajima K, Suchi T, Aozasa K, Matsuzuka F, Kuma K, Tominaga S. Chronic thyroiditis as a risk factor of B-cell lymphoma in the thyroid gland. Jpn J Cancer Res 1985;76:1085-90.
105. Burke JS, Sheibani K, Nathwani BN, Winberg CD, Rappaport H. Monoclonal small (well-differentiated) lymphocytic proliferations of the gastrointestinal tract resembling lymphoid hyperplasia: a neoplasm of uncertain malignant potential. Hum Pathol 1987;18:1238-45.
106. Zulman J, Jaffe R, Talal N. Evidence that the malignant lymphoma of Sjögren's syndrome is a monoclonal B-cell neoplasm. N Engl J Med 1978;299:1215-20.
107. Cleary ML, Galili N, Levy N, Talal N, Sklar J. Detection of lymphoma in Sjögren's syndrome by analysis of immunoglobulin gene rearrangements. In: Talal N, Moutsopoulos HM, Kassan SS, eds. Sjögren's syndrome: clinical and immunological aspects. Berlin: Springer-Verlag, 1987:137-43.
108. Fishleder A, Tubbs R, Hesse B, Levine H. Uniform detection of immunoglobulin-gene rearrangement in benign lymphoepithelial lesion. N Engl J Med 1987;316:1118-21.
109. Jakobiec FA, Neri A, Knowles II DM. Genotypic monoclonality in immunophenotypically polyclonal orbital lymphoid tumors: a model of tumor progression in the lymphoid system. Ophthalmology 1987;94:980-94.
110. Neri A, Jakobiec FA, Pelicci P-G, Dalla-Favera R, Knowles II DM. Immunoglobulin and T cell receptor β chain gene rearrangement analysis of ocular adnexal lymphoid neoplasms: clinical and biologic implications. Blood 1987;70:1519-29.
111. Newland AC, Catovsky D, Linch D, Cawley JC, Beverley P, San Miguel JF, Gordon-Smith EC, Blecher TE, Shahriari S, Varadi S. Chronic T cell lymphocytosis: a review of 21 cases. Brit J Haematol 1984;58:433-446.
112. Reynolds CW, Foon KA. Tγ-lymphoproliferative disease and related disorders in humans and experimental animals: a review of the clinical, cellular, and functional characteristics. Blood 1984;64:1146-58.
113. Loughran TP, Starkebaum G. Large granular lymphocyte leukemia: report of 38 cases and review of the literature. Medicine 1987;66:397-405.
114. Cleary ML, Wood GS, Warnke R, Chao J, Sklar J. Immunoglobulin gene rearrangements in hairy cell leukemia. Blood 1984;64:99-104.
115. Aisenberg AC, Krontiris TG, Mak TW, Wilkes BM. Rearrangement of the gene for the beta chain of the T-cell receptor in T-cell chronic lymphocytic leukemia and related disorders. N Engl J Med 1985;313:529-33.
116. Rambaldi A, Pelicci P-G, Allavena P, Knowles II DM, Rossini S, Bassan R, Barbui T, Dalla-Favera R, Mantovani A. T cell receptor β chain gene rearrangements in lymphoproliferative disorders of large granular lymphocytes/natural killer cells. J Exp Med 1985;162:2156-62.
117. Foa R, Pelicci P-G, Migone N, Laura F, Pizzolo G, Flug F, Knowles II DM, Dalla-Favera R. Analysis of T-cell receptor beta chain (Tβ) gene rearrangements demonstrates the monoclonal nature of T-cell chronic lymphoproliferative disorders. Blood 1986;67:247-50.
118. Berliner N, Duby AD, Linch DC, Murre C, Quertermous T, Knott LJ, Azin T, Newland AC, Lewis DL, Galvin MC, Seidman JG. T cell receptor gene rearrangements define a monoclonal T cell proliferation in patients with T cell lymphocytosis and cytopenia. Blood 1986;67:914-8.
119. Winton EF, Chan WC, Check I, Colenda KW, Bongiovanni KF, Waldmann TA. Spontaneous regression of a monoclonal proliferation of large granular lymphocytes associated with reversal of anemia and neutropenia. Blood 1986;67:1427-32.
120. Koizumi S, Seki H, Tachinami T, Taniguchi M, Matsuda A, Taga K, Nakarai T, Kato E, Taniguchi N, Nakamura H. Malignant clonal expansion of large granular lymphocytes with a Leu-11$^+$, Leu-7$^-$ surface phenotype: In vitro responsiveness of malignant cells to recombinant human interleukin 2. Blood 1986;68:1065-73.
121. Chan WC, Link S, Mawle A, Check I, Brynes RK, Winton EF. Heterogeneity of large granular lymphocyte

proliferations: delineation of two major subtypes. Blood 1986;68:1142-53.
122. Loughran TP Jr, Hammond WP. Adult-onset cyclic neutropenia is a benign neoplasm associated with clonal proliferation of large granular lymphocytes. J Exp Med 1986;164:2089-94.
123. Loiseau P, Divine M, Le Paslier D, Marolleau JP, Farcet JP, Flandrin G, Cohen D, Degos L, Sigaux F, Reyes F. Phenotypic and genotypic heterogeneity in large granular lymphocyte expansion. Leukemia 1987;1:205-9.
124. Hart DNJ, Leahy MF, McKenzie JL, Furley AJ, Carter J, Beard MEJ. Functional analysis of a clonal expansion of Leu-11 positive NK active lymphoid cells. Brit J Haematol 1987;65:277-87.
125. Freimark B, Lanier L, Phillips J, Quertermous T, Fox R. Comparison of T cell receptor gene rearrangements in patients with large granular T cell leukemia and Felty's syndrome. J Immunol 1987;138:1724-9.
126. Grillot-Courvalin C, Vinci G, Tsapis A, Dokhelar M-C, Vainchenker W, Brouet J-C. The syndrome of T8 hyperlymphocytosis: variation in phenotype and cytotoxic activities of granular cells and evaluation of their role in associated neutropenia. Blood 1987;69:1204-10.
127. Landay A, Gebel H, Levin S, Prasthofer E, Pistoia V, Downing J, Grossie C. $CD16^+$ NK lymphoproliferative disorders: cellular and molecular characterization. Nat Immun Cell Growth Regul 1987;6:141-9.
128. Okabe M, Tanaka M, Uehara Y, Matsushima S, Kakinuma M, Abe F, Kobayashi M, Tachibana N, Sakurada K, Saito H, Miyazaki T. Immunological functions and T-cell receptor gene rearrangement of proliferating lymphocytes in a case of T gamma lymphocytosis with neutropenia. Tohoku J Exp Med 1987;151:105-15.
129. Lauria F, Foa R, Migone N, Giubellino MC, Raspadori D, Buzzi M, Casorati G, Gobbi M, Tazzari PL, Tura S. Heterogeneity of large granular lymphocyte proliferations: morphological, immunological and molecular analyses in seven patients. Brit J Haematol 1987;66:187-91.
130. Pelicci P-G, Allavena P, Subar M, Rambaldi A, Pirelli A, Di Bello M, Barbui T, Knowles II DM, Dalla-Favera R, Mantovani A. T cell receptor (α, β, γ) gene rearrangements and expression in normal and leukemic large granular lymphocytes/natural killer cells. Blood 1987;70:1500-8.
131. Foroni L, Foldi J, Matutes E, Catovsky D, O'Connor NJ, Baer R, Forster A, Rabbitts TH, Luzzatto L. α, β and γ T-cell receptor genes: rearrangements correlate with haematological phenotype in T cell leukaemias. Br J Haematol 1987;67:307-18.
132. Chan WC, Dahl C, Waldmann T, Link S, Mawle A, Nicholson J, Bach FH, Bongiovanni K, McCue PA, Winton EF. Large granular lymphocyte proliferation: an analysis of T-cell receptor gene arrangement and expression and the effect of in vitro culture with inducing agents. Blood 1988;71:52-8.
133. Foroni L, Matutes E, Foldi J, Morilla R, Rabbitts TH, Luzzatto L, Catovsky D. T-cell leukemias with rearrangement of the γ but not β T-cell receptor genes. Blood 1988:71:356-62.
134. Oshimi K, Oshimi Y, Akahoshi M, Kobayashi Y, Hirai H, Takaku F, Hattori M, Asano S, Kodo H, Nishinarita S, Lizuka Y, Mizoguche H. Role of T-cell antigens in the cytolytic activities of large granular lymphocytes (LGLs) in patients with LGL lymphocytosis. Blood 1988;71:473-9.
135. Loughran TP Jr, Starkebaum G, Aprile JA. Rearrangement and expression of T-cell receptor genes in large granular lymphocyte leukemia. Blood 1988;71:822-4.
136. Oshimi K, Hoshino S, Takahashi M, Akahoshi M, Saito H, Kobayashi Y, Hirai H, Takaku F, Yahagi N, Oshimi Y, Horie Y, Mizoguchi H. Ti(WT31)-negative, CD3-positive, large granular lymphocyte leukemia with nonspecific cytotoxicity. Blood 1988;71:923-31.
137. Loughran TP Jr, Starkebaum G, Ruscetti FW. Similar rearrangements of T-cell receptor β gene in cell lines and uncultured cells from patients with large granular lymphocyte leukemia. Blood 1988;72:613-5.
138. Imamura N, Kusunoki Y, Kajihara H, Okada K, Kuramoto A. Aggressive natural killer cell leukemia/lymphoma with N901-positive surface phenotype: evidence for the existence of a third lineage in lymphoid cells. Acta Haematol 1988;80:121-8.
139. Bassan R, Rambaldi A, Allavena P, Abbate M, Marini B, Barbui T. Association of large granular lymphocyte/-natural killer cell proliferative disease and second hematologic malignancy. Am J Hematol 1988;29:85-93.
140. Ohno T, Kanoh T, Arita Y, Fujii H, Kuribayashi K, Masuda T, Horiguchi Y, Taniwaki M, Nosaka T, Hatanaka M, Uchino H. Fulminant clonal expansion of large granular lymphocytes: characterization of their morphology, phenotype, genotype, and function. Cancer 1988;62:1918-27.
141. Vie H, Chevalier R, Garand R, Moisan J-P, Praloran V, Devilder M-C, Moreau J-F, Soulillou J-P. Clonal expansion of lymphocytes bearing the $\gamma\delta$ T-cell receptor in a patient with large granular lymphocyte disorder.

Blood 1989;74:285-90.
142. Biondi A, Allavena P, Rossi V, Bassan R, Barbui T, Champagne E, Mak TW, Minden MD, Rambaldi A, Mantovani A. T cell receptor δ gene organization and expression in normal and leukemic natural killer cells. J Immunol 1989;143:1009-14.
143. Ritz J, Campen TJ, Schmidt RE, Royer HD, Hercend T, Hussey RE, Reinherz EL. Analysis of T-cell receptor gene rearrangement and expression in human natural killer clones. Science 1985;228:1540-3.
144. Lanier LL, Cwirla S, Federspiel N, Phillips JH. Human natural killer cells isolated from peripheral blood do not rearrange T cell antigen receptor β chain genes. J Exp Med 1986;163:209-14.
145. Lanier LL, Cwirla S, Phillips JH. Genomic organization of T cell γ genes in human peripheral blood natural killer cells. J Immunol 1986;137:3375-7.
146. Triebel F, Graziani M, Faure F, Jitsukawa S, Hercend T. Cloned human $CD3^-$ lymphocytes with natural killer-like activity do not express nor rearrange T cell receptor gamma genes. Eur J Immunol 1987;17:1209-12.
147. Linch DC, Newland AC, Turnbull AL, Knott LJ, MacWhannel A, Beverley P. Unusual T cell proliferations and neutropenia in rheumatoid arthritis: comparison with classical Felty's syndrome. Scand J Haematol 1984;33:342-50.
148. Sanchez NP, Pittelkow MR, Muller SA, Banks PM, Winkelman RK. The clinicopathologic spectrum of lymphomatoid papulosis: study of 31 cases. J Am Acad Dermatol 1983;8:81-94.
149. Wood GS, Strickler JG, Abel EA, Deneau DG, Warnke RA. Immunohistology of pityriases lichenoides et varioliformis acuta and pityriasis lichenoides chronica: evidence for their interrelationship with lymphomatoid papulosis. J Am Acad Dermatol 1987;16:559-70.
150. Frenkel J, Neijens HJ, Den Hollander JC, Wolvers-Tettero ILM, Van Dongen JJM. Oligoclonal T cell proliferative disorder in combined immunodeficiency. Pediatr Res 1988;24:622-7.
151. Wirt DP, Brooks EG, Vaidya S, Klimpel GR, Waldmann TA, Goldblum RM. Novel T-lymphocyte population in combined immunodeficiency with features of graft-versus-host disease. N Engl J Med 1989;321:370-4.
152. Frizzera G, Moran EM, Rappaport H. Angio-immunoblastic lymphadenopathy with dysproteinaemia. Lancet 1974;i:1070-3.
153. Frizzera G, Moran EM, Rappaport H. Angio-immunoblastic lymphadenopathy: diagnosis and clinical course. Am J Med 1975;59:803-18.
154. Nathwani BN, Rappaport H, Moran EM, Pangalis GA, Kim H. Evolution of immunoblastic lymphoma in angioimmunoblastic lymphadenopathy. Recent Res Cancer Res 1978;64:235-40.
155. Pangalis GA, Moran EM, Nathwani BN, Zelman RJ, Kim H, Rappaport H. Angioimmunoblastic lymphadenopathy: long-term follow-up study. Cancer 1983;52:318-21.
156. Azevedo SJ, Yunis AA. Angioimmunoblastic lymphadenopathy. Am J Hematol 1985;20:301-12.
157. Watanabe S, Sato Y, Shimoyama M, Minato K, Shimosato Y. Immunoblastic lymphadenopathy, angioimmunoblastic lymphadenopathy, and IBL-like T-cell lymphoma: a spectrum of T-cell neoplasia. Cancer 1986;58:2224-32.
158. Mazur EM, Lovett DH, Enriquez RE, Breg WR Jr, Papac RJ. Angioimmunoblastic lymphadenopathy: evolution to a Burkitt-like lymphoma. Am J Med 1979;67:317-24.
159. Klajman A, Yaretzky A, Schneider M, Holoshitz Y, Shneur A, Griffel B. Angioimmunoblastic lymphadenopathy with paraproteinemia: a T- and B-cell disorder. Cancer 1981;48:2433-7.
160. Boros L, Bhaskar AG, D'Souza JP. Monoclonal evolution of angioimmunoblastic lymphadenopathy. Am J Clin Pathol 1981;75:856-60.
161. Tobinai K, Minato K, Ohtsu T, Mukai K, Kagami Y, Miwa M, Watanabe S, Shimoyama M. Clinicopathologic, immunophenotypic, and immunogenotypic analyses of immunoblastic lymphadenopathy-like T-cell lymphoma. Blood 1988;72:1000-6.
162. O'Connor NTJ, Crick JA, Wainscoat JS, Gatter KC, Stein H, Falini B, Mason DY. Evidence for monoclonal T lymphocyte proliferation in angioimmunoblastic lymphadenopathy. J Clin Pathol 1986;39:1229-32.
163. Weiss LM, Strickler JG, Dorfman RF, Horning SJ, Warnke RA, Sklar J. Clonal T-cell populations in angioimmunoblastic lymphadenopathy and angioimmunoblastic lymphadenopathy-like lymphoma. Am J Pathol 1986;122:392-7.
164. Griesser H, Feller A, Lennert K, Minden M, Mak TW. Rearrangement of the β chain of the T cell antigen receptor and immunoglobulin genes in lymphoproliferative disorders. J Clin Invest 1986;78:1179-84.

165. Lipford EH, Smith HR, Pittaluga S, Jaffe ES, Steinberg AD, Cossman J. Clonality of angioimmunoblastic lymphadenopathy and implications for its evolution to malignant lymphoma. J Clin Invest 1987;79:637-42.
166. Suzuki H, Namikawa R, Ueda R, Obata Y, Suchi T, Kikuchi M, Ota K, Takahishi T. Clonal T cell population in angioimmunoblastic lymphadenopathy and related lesions. Jpn J Cancer Res 1987;78:712-20.
167. Hossfeld DK, Höffken K, Schmidt CG, Diedrichs H. Chromosome abnormalities in angioimmunoblastic lymphadenopathy. Lancet 1976;i:198.
168. Kaneko Y, Larson RA, Variakojis D, Haren JM, Rowley JD. Nonrandom chromosome abnormalities in angioimmunoblastic lymphadenopathy. Blood 1982;60:877-87.
169. Gödde-Salz E, Feller AC, Lennert K. Chromosomal abnormalities in lymphogranulomatosis X (LgrX)/angioimmunoblastic lymphadenopathy (AILD). Leukemia Res 1987;11:181-90.
170. Kaneko Y, Maseki N, Sakurai M, Takayama S, Nanba K, Kikuchi M, Frizzera G. Characteristic karyotypic pattern in T-cell lymphoproliferative disorders with reactive "angioimmunoblastic lymphadenopathy with dysproteinemia-type" features. Blood 1988;72:413-21.
171. Savill CM, Delves PJ, Kioussis D, Walker P, Lydyard PM, Colaco B, Shipley M, Roitt IM. A minority of patients with rheumatoid arthritis show a dominant rearrangement of T-cell receptor β chain genes in synovial lymphocytes. Scand J Immunol 1987;25:629-35.
172. Hafler DA, Duby AD, Lee SJ, Benjamin D, Seidman JG, Weiner HL. Oligoclonal T lymphocytes in the cerebrospinal fluid of patients with multiple sclerosis. J Exp Med 1988;167:1313-22.
173. Katzin WE, Fishleder AJ, Tubbs RR. Investigation of the clonality of lymphocytes in Hashimoto's thyroiditis using immunoglobulin and T-cell receptor gene probes. Clin Immunol Immunopathol 1989;51:264-74.
174. Foa R, Migone N, Saitta M, Fierro MT, Giubellino MC, Lusso P, Cordero di Montezemolo L, Miniero R, Lauria F. Different stages of B cell differentiation in non-T acute lymphoblastic leukemia. J Clin Invest 1984;74:1756-63.
175. Kitchingman GR, Mirro J, Stass S, Rovigatti U, Melvin SL, Williams DL, Raimondi SC, Murphy SB. Biologic and prognostic significance of the presence of more than two μ heavy-chain genes in childhood acute lymphoblastic leukemia of B precursor cell origin. Blood 1986;67:698-703.
176. Raghavachar A, Bartram CR, Ganser A, Heil G, Kleihauer E, Kubanek B. Acute undifferentiated leukemia: implications for cellular origin and clonality suggested by analysis of surface markers and immunoglobulin gene rearrangement. Blood 1986;68:658-62.
177. Rechavi G, Brok-Simoni F, Katzir N, Mandel M, Umiel T, Stark B, Zaizov R, Ben-Bassat I, Ramot B. More than two immunoglobulin heavy chain J region genes in the majority of infant leukemia. Leukemia 1988;2:347-50
178. Bird J, Galili N, Link M, Stites D, Sklar J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukemia. J Exp Med 1988;168:229-45.
179. Ludwig W-D, Bartram CR, Harbott J, Köller U, Haas OA, Hansen-Hagge T, Heil G, Seibt-Jung H, Teichmann JV, Ritter J, Knapp W, Gadner H, Thiel E, Riehm H. Phenotypic and genotypic heterogeneity in infant acute leukemia. I. Acute lymphoblastic leukemia. Leukemia 1989;3:431-9.
180. Katz F, Ball L, Gibbons B, Chessells J. The use of DNA probes to monitor minimal residual disease in childhood acute lymphoblastic leukaemia. Br J Haematol 1989;73:173-80.
181. Sklar J, Cleary ML, Thielemans K, Gralow J, Warnke R, Levy R. Biclonal B-cell lymphoma. N Engl J Med 1984;311:20-7.
182. Raffeld M, Neckers L, Longo DL, Cossman J. Spontaneous alteration of idiotype in a monoclonal B-cell lymphoma: escape from detection by anti-idiotype. N Engl J Med 1985;312:1653-8.
183. Meeker T, Lowder J, Cleary ML, Stewart S, Warnke R, Sklar J, Levy R. Emergence of idiotype variants during treatment of B-cell lymphoma with anti-idiotype antibodies. N Engl J Med 1985;312:1658-65.
184. Cleary ML, Meeker TC, Levy S, Lee E, Trela M, Sklar J, Levy R. Clustering of extensive somatic mutations in the variable region of an immunoglobulin heavy chain gene from a human B cell lymphoma. Cell 1986;44:97-106.
185. Carroll WL, Lowder JN, Streifer R, Warnke R, Levy S, Levy R. Idiotype variant cell populations in patients with B cell lymphoma. J Exp Med 1986;164:1566-80.
186. Cleary ML, Galili N, Trela M, Levy R, Sklar J. Single cell origin of bigenotypic and biphenotypic B cell proliferations in human follicular lymphomas. J Exp Med 1988;167:582-97.

187. De Jong D, Voetdijk BMH, Van Ommen GJB, Kluin PM. Alterations in immunoglobulin genes reveal the origin and evolution of monotypic and bitypic B cell lymphomas. Am J Pathol 1989;134:1233-42.
188. Dow LW, Martin P, Moohr J, Greenberg M, Macdougall LG, Najfeld V, Fialkow PJ. Evidence for clonal development of childhood acute lymphoblastic leukemia. Blood 1985;66:902-7.
189. Williams DL, Look AT, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S. New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia. Cell 1984;36:101-9.
190. Ahuja HG, Cline MJ. Review: genetic and cytogenetic changes in acute lymphoblastic leukemia. Med Oncol Tumor Pharmacother 1988;5:211-22.
191. Pui C-H, Raskind WH, Kitchingman GR, Raimondi SC, Behm FG, Murphy SB, Crist WM, Fialkow PJ, Williams DL. Clonal analysis of childhood acute lymphoblastic leukemia with "cytogenetically independent" cell populations. J Clin Invest 1989;83:1971-7.
192. Williams DL, Tsiatis A, Brodeur GM, Look AT, Melvin SL, Bowman WP, Kalwinsky DK, Rivera G, Dahl GV. Prognostic importance of chromosome number in 136 untreated children with acute lymphoblastic leukemia. Blood 1982;60:864-71.
193. Williams DL, Harber J, Murphy SB, Look AT, Kalwinsky DK, Rivera G, Melvin SL, Stass S, Dahl GV. Chromosomal translocations play a unique role in influencing prognosis in childhood acute lymphoblastic leukemia. Blood 1986;68:205-12.
194. Croce CM, Shander M, Martinis J, Cicurel L, D'Ancona GG, Dolby TW, Koprowski H. Chromosomal location of the genes for human immunoglobulin heavy chains. Proc Natl Acad Sci USA 1979;76:3416-9.
195. Kirsch IR, Morton CC, Nakahara K, Leder Ph. Human immunoglobulin heavy chain genes map to a region of translocations in malignant B lymphocytes. Science 1982;216:301-3.
196. Reth MG, Jackson S, Alt FW. VHDJH formation and DJH replacement during pre-B differentiation: non-random usage of gene segments. EMBO J 1986;5:2131-8.
197. Reth M, Gehrmann P, Petrac E, Wiese P. A novel VH to VHDJH joining mechanism in heavy-chain-negative (null) pre-B cells results in heavy-chain production. Nature 1986;322:840-2.
198. Kleinfield R, Hardy RR, Tarlinton D, Dangl J, Herzenberg LA, Weigert M. Recombination between an expressed immunoglobulin heavy-chain gene and a germline variable gene segment in a Ly 1^+ B-cell lymphoma. Nature 1986;322:843-6.
199. Epplen JT, Bartels F, Becker A, Nerz G, Prester M, Rinaldy A, Simon MM. Change in antigen specificity of cytotoxic T lymphocytes is associated with the rearrangement and expression of a T-cell receptor β-chain gene. Proc Natl Acad Sci USA 1986;83:4441-5.
200. Fondell JD, Marolleau J-P, Primi D, Marcu KB. On the mechanism of non-allelically excluded Vα-Jα T cell receptor secondary rearrangements in a murine T cell lymphoma. J Immunol 1990;144:1094-103.
201. Siekevitz M, Kocks C, Rajewsky K, Dildrop R. Analysis of somatic mutation and class switching in naive and memory B cells generating adoptive primary and secondary responses. Cell 1987;48:757-70.
202. Rajewsky K, Förster I, Cumano A. Evolutionary and somatic selection of the antibody repertoire in the mouse. Science 1987;238:1088-94.
203. Nieuwenhuis P, Opstelten D. Functional anatomy of germinal centers. Am J Anat 1984;170:421-35.
204. MacLennan ICM, Gray D. Antigen-driven selection of virgin and memory B cells. Immunol Rev 1986;91:61-85.
205. Webb CF, Cooper MD, Burrows PD, Griffin JA. Immunoglobulin gene rearrangements and deletions in human Epstein-Barr virus-transformed cell lines producing different IgG and IgA subclasses. Proc Natl Acad Sci USA 1985;82:5495-9.
206. Borzillo GV, Cooper MD, Kubagawa H, Landay A, Burrows PD. Isotype switching in human B lymphocyte malignancies occurs by DNA deletion: evidence for nonspecific switch recombination. J Immunol 1987;139:1326-35.
207. Miller WJ, Shapiro RS, Gonzalez-Sarmiento R, Kersey JH. Molecular genetic rearrangements distinguish pre- and post-bone marrow transplantation lymhoproliferative processes. Blood 1987;70:882-5.
208. Siegelman MH, Cleary ML, Warnke R, Sklar J. Frequent biclonality and Ig gene alterations among B cell lymphomas that show multiple histologic forms. J Exp Med 1985;161:850-63.
209. Wright JJ, Poplack DG, Bakhshi A, Reaman G, Cole D, Jensen JP, Korsmeyer SJ. Gene rearrangements as markers of clonal variation and minimal residual disease in acute lymphoblastic leukemia. J Clin Oncol

1987;5:735-41.
210. Raghavachar A, Thiel E, Bartram CR. Analyses of phenotype and genotype in acute lymphoblastic leukemias at first presentation and in relapse. Blood 1987;70:1079-83.
211. Raghavachar A, Ludwig W-D, Bartram CR. Clonal variation in childhood acute lymphoblastic leukaemia at early and late relapse detected by analyses of phenotype and genotype. Eur J Pediatr 1988;147:503-7.
212. Kowalczyk JR, Grossi M, Sandberg AA. Cytogenetic findings in childhood acute lymphoblastic leukemia. Cancer Genet Cytogenet 1985;15:47-64.
213. Pui C-H, Raimondi SC, Behm FG, Ochs J, Furman WL, Bunin NJ, Ribeiro RC, Tinsley PA, Mirro J. Shifts in blast cell phenotype and karyotype at relapse of childhood lymphoblastic leukemia. Blood 1986;68:1306-10.
214. Yunis JJ, Oken MM, Kaplan ME, Ensrud KM, Howe RR, Theologides A. Distinctive chromosomal abnormalities in histologic subtypes of non-Hodgkin's lymphoma. N Engl J Med 1982;307:1231-6.
215. Bloomfield CD, Arthur DC, Frizzera G, Levine EG, Peterson BA, Gajl-Peczalska KJ. Nonrandom chromosome abnormalities in lymphoma. Cancer Res 1983;43:2975-84.
216. Levine EG, Arthur DC, Frizzera G, Peterson BA, Hurd DD, Bloomfield CD. There are differences in cytogenetic abnormalities among histologic subtypes of the non-Hodgkin's lymphomas. Blood 1985;66:1414-22.
217. Koduru PRK, Filippa DA, Richardson ME, Jhanwar SC, Chaganti SR, Koziner B, Clarkson BD, Lieberman PH, Chaganti RSK. Cytogenetic and histologic correlations in malignant lymphoma. Blood 1987;69:97-102.
218. Tsujimoto Y, Cossman J, Jaffe E, Croce CM. Involvement of the *bcl-2* gene in human follicular lymphoma. Science 1985;228:1440-3.
219. Bakhshi A, Jensen JP, Goldman P, Wright JJ, McBride OW, Epstein AL, Korsmeyer SJ. Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. Cell 1985;41:899-906.
220. Weiss LM, Warnke RA, Sklar J, Cleary ML. Molecular analysis of the t(14;18) chromosomal translocation in malignant lymphomas. N Engl J Med 1987;317:1185-9.
221. Raffeld M, Wright JJ, Lipford E, Cossman J, Longo DL, Bakhshi A, Korsmeyer SJ. Clonal evolution of t(14;18) follicular lymphomas demonstrated by immunoglobulin genes and the 18q21 major breakpoint region. Cancer Res 1987;47:2537-42.
222. Bertoli LF, Kubagawa H, Borzillo GV, Mayumi M, Prchal JT, Kearney JF, Durant JR, Cooper MD. Analysis with antiidiotype antibody of a patient with chronic lymphocytic leukemia and a large cell lymphoma (Richter's syndrome). Blood 1987;70:45-50.
223. McNally L, Jakobiec FA, Knowles II DM. Clinical, morphologic, immunophenotypic and molecular genetic analysis of bilateral ocular adnexal lymphoid neoplasms in 17 patients. Am J Ophthalmol 1987;103:555-68.
224. Neri A, Jakobiec FA, Pelicci P-G, Dalla-Favera R, Knowles II DM. Immunoglobulin and T cell receptor β chain gene rearrangement analysis of ocular adnexal lymphoid neoplasms: clinical and biological implications. Blood 1987;70:1519-29.
225. De Jong D, Voetdijk BMH, Beverstock GC, Van Ommen GJB, Willemze R, Kluin PM. Activation of the *c-myc* oncogene in a precursor-B-cell blast crisis of follicular lymphoma, presenting as composite lymphoma. N Engl J Med 1988;318:1373-8.
226. Michiels JJ, Van Dongen JJM, Hagemeijer A, Sonneveld P, Ploemacher RE, Adriaansen HJ, Van der Kwast ThH, Brederoo P, Abels J. Richter's syndrome with identical immunoglobulin gene rearrangements in the chronic lymphocytic leukemia and the supervening non-Hodgkin lymphoma. Leukemia 1989;3:819-24.
227. Van Dongen JJM, Hooijkaas H, Michiels JJ, Grosveld G, De Klein A, Van der Kwast ThH, Prins MEF, Abels J, Hagemeijer A. Richter's syndrome with different immunoglobulin light chains and different heavy chain gene rearrangements. Blood 1984;64:571-5.
228. Giardina SL, Schroff RW, Woodhouse CS, Golde DW, Oldham RK, Cleary ML, Sklar J, Pritikin N, Foon KA. Detection of two distinct malignant B cell clones in a single patient using anti-idiotype monoclonal antibodies and immunoglobulin gene rearrangement. Blood 1985;66:1017-21.
229. Downing JR, Grossi CE, Smedberg CT, Burrows PD. Diffuse large cell lymphoma in a patient with hairy cell leukemia: immunoglobulin gene analysis reveals separate clonal origins. Blood 1986;67:739-44.
230. McDonnell JM, Beschorner WE, Staal SP, Spivak JL, Mann RB. Richter's syndrome with two different B-cell clones. Cancer 1986;58:2031-37.

231. Ben-Ezra J, Winberg CD, Wu A, Sheibani K, Rappaport H. Concurrent presence of two clonal populations in small lymphocytic lymphoma of the lung. Hum Pathol 1987;18:399-402.
232. Ostrowski M, Minden M, Wang C, Bailey D. Immunophenotypic and gene probe analysis of a case of Richter's syndrome. Am J Clin Pathol 1989;91:215-21.
233. Hu E, Weiss LM, Warnke R, Sklar J. Non-Hodgkin's lymphoma containing both B and T cell clones. Blood 1987;70:287-92.
234. Lortholary P, Boiron M, Ripault P, Levy JP, Manus A, Bernard J. Leucémie lymphoïde chronique secondairement associée a une réticulopathie maligne syndrome de Richter. Nouv Rev Fr Hematol 1964;5:621-44.
235. Trump DL, Mann RB, Phelps R, Roberts H, Conley CL. Richter's syndrome: diffuse histiocytic lymphoma in patients with chronic lymphocytic leukemia: a report of five cases and review of the literature. Am J Med 1980;68:539-48.
236. Richter MN. Generalized reticular cell sarcoma of lymph nodes associated with lymphatic leukemia. Am J Pathol 1928;IV:285-92.
237. Foucar K, Rydell RE. Richter's syndrome in chronic lymphocytic leukemia. Cancer 1980;46:118-34.
238. Delsol G, Laurent G, Kuhlein E, Familiades J, Rigal F, Pris J. Richter's syndrome: evidence for the clonal origin of the two proliferations. Am J Clin Pathol 1981;76:308-15.
239. Splinter TAW, Bom-Van Noorloos A, Van Heerde P. CLL and diffuse histiocytic lymphoma in one patient: clonal proliferation of two different B cells. Scand J Haematol 1978;20:29-36.
240. Greaves MF, Furley AJW, Chan LC, Ford AM, Molgaard HV. Inappropriate rearrangement of immunoglobulin and T-cell receptor genes. Immunol Today 1987;8:115-6.
241. Furley AJW, Chan LC, Mizutani S, Ford AM, Weilbaecher K, Pegram SM, Greaves MF. Lineage specificity of rearrangement and expression of genes encoding the T cell receptor-T3 complex and immunoglobulin heavy chain in leukemia. Leukemia 1987;1:644-52.
242. Lennert K, Mohri N, Stein H, Kaiserling E. The histopathology of malignant lymphoma. Br J Haematol 1975;31S:193-203.
243. Lennert K, Feller AC. Morphology and immunohistology of T cell lymphomas. In: Quaglino D, Hayhoe FGJ, eds. The cytobiology of leukaemias and lymphomas. New York: Raven Press, 1985:81-90.
244. O'Connor NTJ, Feller AC, Wainscoat JS, Gatter KC, Pallesen G, Stein H, Lennert K, Mason DY. T-cell origin of Lennert's lymphoma. Br J Haematol 1986;64:521-8.
245. Griesser H, Feller A, Lennert K, Tweedale M, Messner HA, Zalcberg J, Minden MD, Mak TW. The structure of the T cell gamma chain gene in lymphoproliferative disorders and lymphoma cell lines. Blood 1986;68:592-4.
246. Tkachuk DC, Griesser H, Takihara Y, Champagne E, Minden M, Feller AC, Lennert K, Mak TW. Rearrangement of T-cell δ locus in lymphoproliferative disorders. Blood 1988;72:353-7.
247. Stein H, Mason DY, Gerdes J, O'Connor N, Wainscoat J, Pallesen G, Gatter K, Falini B, Delsol G, Lemke H, Schwarting R, Lennert K. The expression of the Hodgkin's disease associated antigen Ki-1 in reactive and neoplastic lymphoid tissue: evidence that Reed-Sternberg cells and histiocytic malignancies are derived from activated lymphoid cells. Blood 1985;66:848-58.
248. O'Connor NTJ, Stein H, Gatter KC, Wainscoat JS, Crick J, Al Saati T, Falini B, Delsol G, Mason DY. Genotypic analysis of large cell lymphomas which express the Ki-1 antigen. Histopathology 1987;11:733-40.
249. Herbst H, Tippelmann G, Anagnostopoulos I, Gerdes J, Schwarting R, Boehm T, Pileri S, Jones DB, Stein H. Immunoglobulin and T-cell receptor gene rearrangements in Hodgkin's disease and Ki-1-positive anaplastic large cell lymphoma: dissociation between phenotype and genotype. Leuk Res 1989;13:103-16.
250. Knowles II DM, Neri A, Pelicci PG, Burke JS, Wu A, Winberg CD, Sheibani K, Dalla-Favera R. Immunoglobulin and T-cell receptor β-chain gene rearrangement analysis of Hodgkin's disease: implications for lineage determination and differential diagnosis. Proc Natl Acad Sci USA 1986;83:7942-6.
251. Weiss LM, Strickler JG, Hu E, Warnke RA, Sklar J. Immunoglobulin gene rearrangements in Hodgkin's disease. Hum Pathol 1986;17:1009-14.
252. O'Conner NTJ, Crick JA, Gatter KC, Mason DY, Falini B, Stein HS. Cell lineage in Hodgkin's disease. Lancet 1987;i:158.
253. Griesser H, Feller AC, Mak TW, Lennert K. Clonal rearrangements of T-cell receptor and immunoglobulin

genes and immunophenotypic antigen expression in different subclasses of Hodgkin's disease. Int J Cancer 1987;40:157-60.

254. Sundeen J, Lipford E, Uppenkamp M, Sussman E, Wahl L, Raffeld M, Cossman J. Rearranged antigen receptor genes in Hodgkin's disease. Blood 1987;70:96-103 and Errata 1987;70:893-4.
255. Brinker MGL, Poppema S, Buys CHCM, Timens W, Osinga J, Visser L. Clonal immunoglobulin gene rearrangements in tissues involved by Hodgkin's disease. Blood 1987;70:186-91.
256. Linden MD, Fishleder AJ, Katzin WE, Tubbs RR. Absence of B-cell or T-cell clonal expansion in nodular, lymphocyte predominant Hodgkin's disease. Hum Pathol 1988;19:591-4.
257. Roth MS, Schnitzer B, Bingham EL, Harnden CE, Hyder DM, Ginsburg D. Rearrangement of immunoglobulin and T-cell receptor genes in Hodgkin's disease. Am J Pathol 1988;131:331-8.
258. Raghavachar A, Binder T, Bartram CR. Immunoglobulin and T-cell receptor gene rearrangements in Hodgkin's disease. Cancer Res 1988;48:3591-4.
259. Drexler HG, Jones DB, Diehl V, Minowada J. Is the Hodgkin cell a T- or B-lymphocyte? Recent evidence from geno- and immunophenotypic analysis and *in-vitro* cell lines. Hematol Oncol 1989;7:95-113.
260. Weiss LM, Strickler JG, Warnke RA, Purtilo DT, Sklar J. Epstein-Barr viral DNA in tissues of Hodgkin's disease. Am J Pathol 1987;129:86-91.
261. Meyers FJ, Miller CH, Lewis JP. The resolution of phenotypic ambiguity in a case of human leukemia using cytogenetics and immunoglobulin gene rearrangement. Am J Clin Pathol 1986;85:372-4.
262. Erlich HA, Gelfand DH, Saiki RK. Specific DNA amplification. Nature 1988;331:461-2.
263. Bell J. The polymerase chain reaction. Immunol Today 1989;10:351-5.
264. Eisenstein BI. The polymerase chain reaction: a new method of using molecular genetics for medical diagnosis. N Engl J Med 1990;322:178-83.
265. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 1987;237:175-8.
266. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ. Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 1988;85:4869-73.
267. Morgan GJ, Hughes T, Janssen JWG, Gow J, Guo A-P, Goldman JM, Wiedemann LM, Bartram CR. Polymerase chain reaction for detection of residual leukaemia. Lancet 1989;i:928-9.
268. D'Auriol L, Macintyre E, Galibert F, Sigaux F. In vitro amplification of T cell γ gene rearrangements: a new tool for the assessment of minimal residual disease in acute lymphoblastic leukemias. Leukemia 1989;3:155-8.
269. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, Tsujimoto Y, Caton AJ, Rovera G. Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementary-determining region (CDR-III)-specific probes. Proc Natl Acad Sci USA 1989;86:5123-7.
270. Hansen-Hagge TE, Yokota S, Bartram CR. Detection of minimal residual disease in acute lymphoblastic leukemia by in vitro amplification of rearranged T-cell receptor δ chain sequences. Blood 1989;74:1762-7.

CHAPTER 7

GENERAL DISCUSSION

CHAPTER 7

GENERAL DISCUSSION

The extensive immunologic marker studies of normal immature and mature T cells as well as the studies on the rearrangement and expression of T cell receptor (TcR) genes in T cell acute lymphoblastic leukemias (T-ALL) have given more insight into the various step-wise processes which occur during T cell differentiation. Especially, detailed information has been obtained about the expression of the TcR-CD3 complex during T cell development. In addition, analysis of TcR-$\alpha\beta$ and TcR-$\gamma\delta$ expression by blood T lymphocytes in healthy children as well as infants with a hypoplastic or aplastic thymus (DiGeorge anomaly) has provided evidence for the existence of an extrathymic TcR-$\gamma\delta$ pathway, while the development of TcR-$\alpha\beta^+$ cells is highly thymus-dependent. Detailed experimental data on human T cell differentiation and the interpretation of these data are described in Chapter 4.

The experience and knowledge concerning immunologic marker analysis of normal and malignant T cells was extended to other hematopoietic cells and their malignant counterparts (Chapter 2). This allowed us to compose optimal panels of immunologic markers for immunophenotyping of the various types of leukemias and non-Hodgkin lymphomas (NHL), which is important for a detailed and reproducible classification of these malignancies (Chapters 5.1 and 5.2).

Similarly, the experience with the analysis of TcR genes was extended to the analysis of immunoglobulin (Ig) genes (Chapter 3.3). The great majority of lymphoid malignancies of B and T cell origin have clonally rearranged Ig and TcR genes, respectively. Also cross-lineage Ig and TcR gene rearrangements occur. This especially concerns TcR gene rearrangements in precursor B cell acute lymphoblastic leukemias (precursor B-ALL), Ig heavy chain (IgH) gene rearrangements in T-ALL and Ig and/or TcR gene rearrangements in acute myeloid leukemias (AML) (Chapter 6.2). This information can be used for diagnostic purposes, because Southern blot analysis of Ig and TcR genes is an excellent tool for the detection of clonality. The diagnostic applications are as follows: discrimination between polyclonal and monoclonal lymphoproliferations; detection of two or more subclones within a malignancy at diagnosis; detection of clonal evolution at relapse; analysis of the clonal origin of two lymphoid malignancies in a single patient; and analysis of the differentiation lineage of a malignancy. Although virtually all malignancies are clonal proliferations, it should be noticed that a clonal cell population does not always represent a malignancy (Chapter 6.3). In the majority of patients with a lymphoproliferative disease morphological techniques and immunologic marker analysis give sufficient information, but in 1 to 5% of cases gene rearrangement studies are necessary to make a correct diagnosis. The application of Ig and TcR gene analysis for diagnostic purposes should be restricted to these cases, because the Southern blot technique is expensive and time-consuming.

Appropriate immunologic marker analysis and Southern blot analysis of Ig and TcR genes can give important complementary information in addition to the information from routine cytomorphology. The combined morphological, immunophenotypic and genotypic information allows a more accurate diagnosis in patients with lymphoproliferative diseases or related disorders. This is not only important at initial diagnosis, but also during follow-up of these patients. However, the detection of low numbers of malignant cells is a major problem at diagnosis and during follow-up (1,2). During treatment and even after withdrawal of therapy it remains uncertain whether or not malignant cells are still present. This is especially true in childhood ALL, because these leukemias may relapse up to five years or more after initial diagnosis and subsequent treatment (1,2). Therefore, we have put much effort in designing techniques for detection of minimal (residual) disease in ALL.

DETECTION OF MINIMAL RESIDUAL DISEASE IN ALL

The detection limit of conventional morphological techniques is not lower than 1 to 5% (i.e. 1 to 5 malignant cells between 100 normal cells) since small numbers of malignant cells are difficult to discriminate from normal cells (1,2). Also the application of immunologic marker analysis for the detection of low numbers of malignant cells is hampered by the presence of normal counterparts which express the same markers. However, in specific cases it is possible to detect minimal disease, based on the assumption that the presence of positive cells outside their normal "homing areas" is indicative of malignancy (Chapter 5.3). In this way the enzyme terminal deoxynucleotidyl transferase (TdT) can be used as marker for the detection of ALL cells in cerebrospinal fluid (3-5). This allows the early detection (or exclusion) of central nervous system leukemia which is a severe complication in ALL patients (Chapter 5.4). Another example is the detection of extremely low numbers of malignant cells in bone marrow (BM) and peripheral blood (PB) of patients with a TdT^+ T cell malignancy by use of a double immunofluorescence (IF) staining technique. This is based on the observation that certain T cell $marker^+/TdT^+$ cells normally occur in the thymus only and not in extrathymic locations such as BM and PB (Chapter 5.3).

Detection of minimal residual disease in TdT^+ T cell malignancies

Nearly all T-ALL and most T cell lymphoblastic lymphomas (T-LBL) express TdT as well as the pan-T cell markers CD2, cytoplasmic CD3 (CyCD3), CD5 and CD7. Many of these also express additional T cell markers such as CD1, surface membrane CD3 (SmCD3), CD4 and/or CD8. Most cortical thymocytes express the T cell $marker^+/TdT^+$ phenotype, but in extrathymic locations like BM and PB such cells are extremely rare. If they occur, they express the CD2 and/or CD7 antigens, but not CyCD3, CD5 or other T cell markers (Chapters 4.2 and 5.3 and refs. 6-11). To determine the sensitivity of the double IF staining technique for a T cell marker and TdT, we have performed six dilution experiments, in which normal thymocytes (n=1), TdT^+ T-LBL cells (n=2) or T-ALL cells (n=3) were mixed in various proportions with normal PB cells (8). These dilution experiments demonstrated that the double IF staining technique allows the detection of one T cell $marker^+/TdT^+$ cell among 10,000 or even 100,000

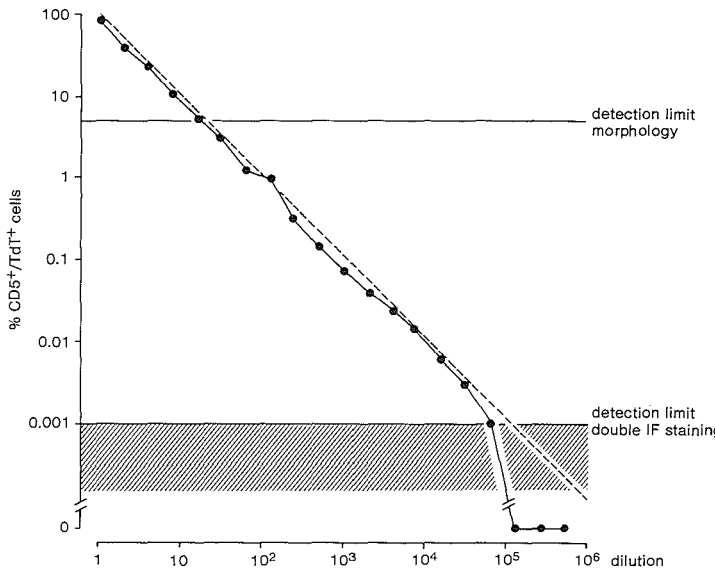


Figure 1. Determination of the sensitivity of the T cell marker/TdT double IF staining technique. T-ALL cells were diluted with normal PB cells and subjected to a CD5/TdT double IF staining. The labeled cells in the cytocentrifuge preparations were evaluated by fluorescence microscopy. It appeared to be possible to detect one $CD5^+/TdT^+$ cell between 100,000 PB cells.

normal cells (Figure 1).

Based on these data it can be concluded that the T cell marker/TdT double IF staining technique is a valuable tool for the detection of low numbers of malignant cells in patients with a TdT^+ T cell malignancy (Chapter 5.3 and refs. 11-13). In November 1982 we have started to use this double IF staining technique for monitoring of BM and PB samples of patients with a TdT^+ T cell malignancy. The results of the first three years of this follow-up study are summarized in Chapter 5.3. Until now (May 1990) the double IF staining technique has been used to analyze BM and PB samples from 24 T-ALL patients and four T-LBL patients at diagnosis as well as during follow-up (generally at 4 to 8 week intervals).

At diagnosis the four T-LBL patients had a stage I or stage II disease according to cytomorphological criteria. However, by use of the double IF staining technique $CD5^+/TdT^+$ cells were detected in the BM and PB of each of the T-LBL patients, which indicated that they in fact had a stage IV disease. These findings confirm earlier data suggesting that T-LBL has a high tendency to disseminate and explain why local treatment is insufficient in most T-LBL patients (14).

During follow-up of the 28 patients, nine T-ALL patients developed one or two BM relapses (total: n=11). Except for one, all BM relapses were detected 5 to 21 weeks (median: 15 weeks) earlier than by use of conventional morphology. The monitoring of the $CD5^+/TdT^+$ cells in PB of these patients is illustrated in Figure 2. The percentages of $CD5^+/TdT^+$ cells in PB appeared to parallel those in BM, suggesting that T-ALL patients can be monitored by analysis of their PB only. One BM relapse was not predicted, probably because no BM or PB samples could

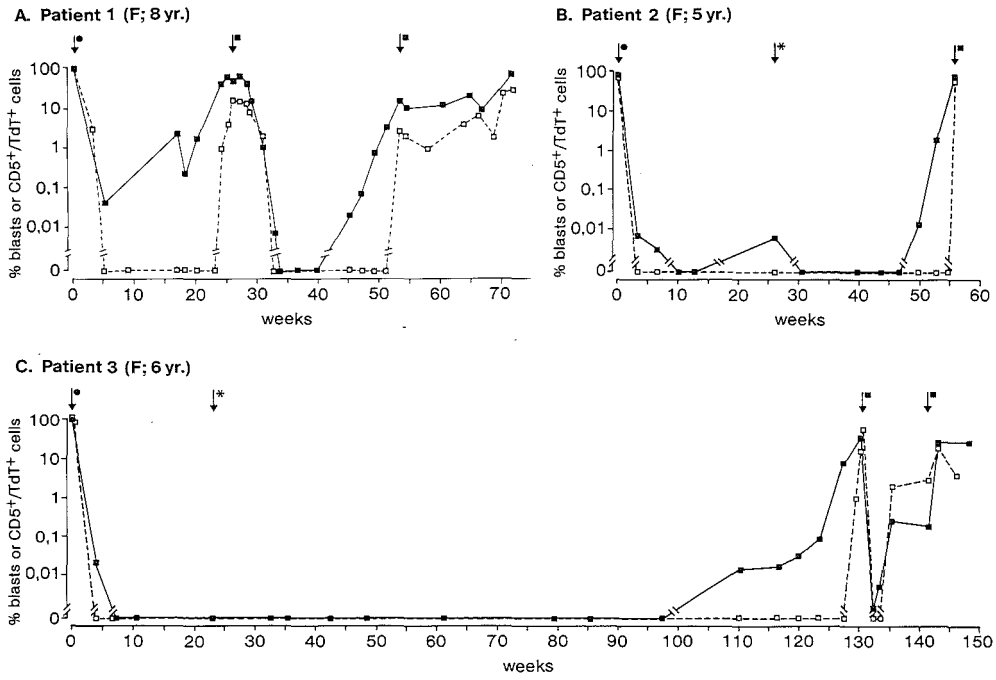


Figure 2. Follow-up of three T-ALL patients by analysis of PB samples using cytomorphology and the CD5/TdT double IF staining. Symbols: *solid squares*, $CD5^+/TdT^+$ cells; *open squares*, blast cells; ↓●, induction therapy; ↓■, reinduction therapy; ↓*, diagnosis of CNS leukemia and subsequent intrathecal therapy. In the three patients the relapses could be predicted 5 to 21 wk earlier than by use of conventional cytomorphology.

be analyzed during the 15-week period before relapse. In the BM and PB of the other 15 T-ALL patients and the four T-LBL patients no $CD5^+/TdT^+$ cells were detected during a follow-up period of 7 to 89 months (median: 52 months). These patients are still in remission according to cytomorphological and clinical criteria.

Our data indicate that double IF staining for a T cell marker and TdT represents a sensitive and specific technique for the follow-up of patients with a TdT^+ T cell malignancy and they suggest that monitoring of PB only is sufficient for early detection of relapse. The time periods between the first immunologic and first morphological detection of malignant cells indicate that the monitoring should be performed at 4 to 5 weeks intervals.

Detection of minimal residual disease by use of the polymerase chain reaction

Immunologic marker analysis cannot efficiently be used for the detection of minimal residual disease in patients with precursor B-ALL, because normal counterparts of such leukemic cells occur in relatively high frequencies in BM and PB, especially during the period of BM regeneration after withdrawal of cytotoxic treatment. However, recent reports indicate that the polymerase chain reaction (PCR) technique may be useful for the detection of minimal residual

disease via the amplification of the junctional regions of rearranged Ig and TcR genes (15-17). This is based on the fact that the junctional regions of rearranged Ig and TcR genes can vary enormously due to the differential ways of joining of V, (D) and J gene segments and the insertion of additional nucleotides at the joining sites (Chapter 3.2). The junctional regions probably differ in each lymphocyte (clone) and therefore also in each lymphoid leukemia.

The specificity of the PCR-mediated amplification of junctional regions is based on the use of two synthetic oligonucleotides, which specifically recognize sequences on opposite strands that flank the DNA region of interest, i.e. the junctional region (18-21). These oligonucleotides function as primers for the DNA polymerase and are oriented with their 3′ ends pointing towards each other. In this way the polymerase-mediated DNA synthesis proceeds across the junctional region. An example is given in Figure 3, where the Vδ1 gene segment is joined to the Jδ1 gene segment via a junctional region which probably contains one or more Dδ gene segments as well as additional nucleotides at the joining sites of the gene segments. Two sets of primers are indicated in this figure: an outer set of a Vδ1-5′ oligo in combination with a Jδ1-3′ oligo, and an inner set of a Vδ1-3′ oligo and a Jδ1-5′ oligo. These sets of primers can be used next to each other or sequentially.

The PCR procedure involves repeated cycles of heat denaturation of the genomic DNA, annealing of the oligonucleotide primers to their complementary sequences, and extension of the annealed primers by use of the *Taq* polymerase (18-21). The extension product of one primer can serve as template for the other primer in the next PCR cycles, resulting in an exponential amplification of the junctional region (18-21). After this amplification, the PCR product can be detected in a dot blot or Southern blot by use of a leukemia-specific junctional region probe which has to be designed specifically for each patient (15-17).

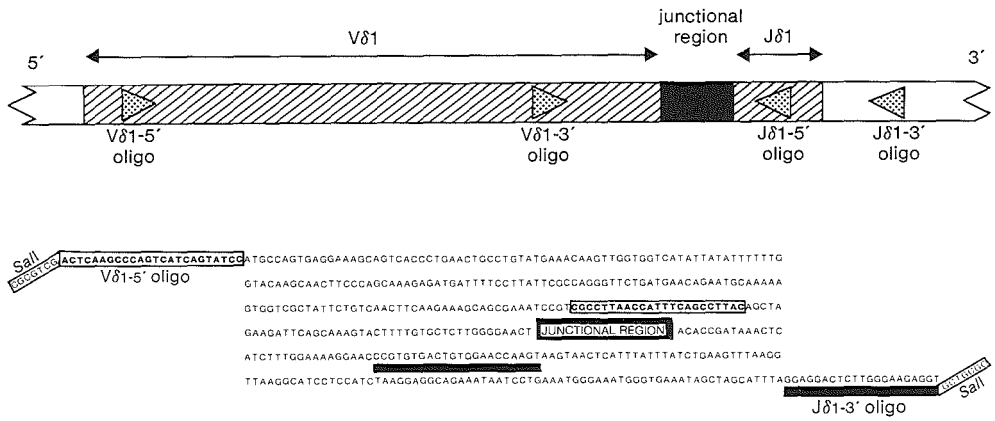


Figure 3. Schematic diagram of the Vδ1 gene segment, joined to the Jδ1 gene segment via a fictitious junctional region which may contain one or more Dδ gene segments as well as additional nucleotides at the joining sites. Four oligonucleotide primers for PCR-mediated amplification of the junctional region are indicated. *Upper*: The Vδ1-5′ and Vδ1-3′ oligonucleotides and the Jδ1-3′ and Jδ1-5′ oligonucleotides are indicated as dotted arrows, which point to the right and to the left, respectively. *Lower*: Nucleotide sequence of the rearranged Vδ1-Jδ1 gene segments. The Vδ1-5′ and Vδ1-3′ oligonucleotides are boxed, while the Jδ1-3′ and Jδ1-5′ oligonucleotides are indicated by bars, which are complementary to the opposite nucleotide sequences. The *SalI* restriction site at the 5′ ends of the Vδ1-5′ and Jδ1-3′ oligonucleotide primers can be used for cloning of the PCR product.

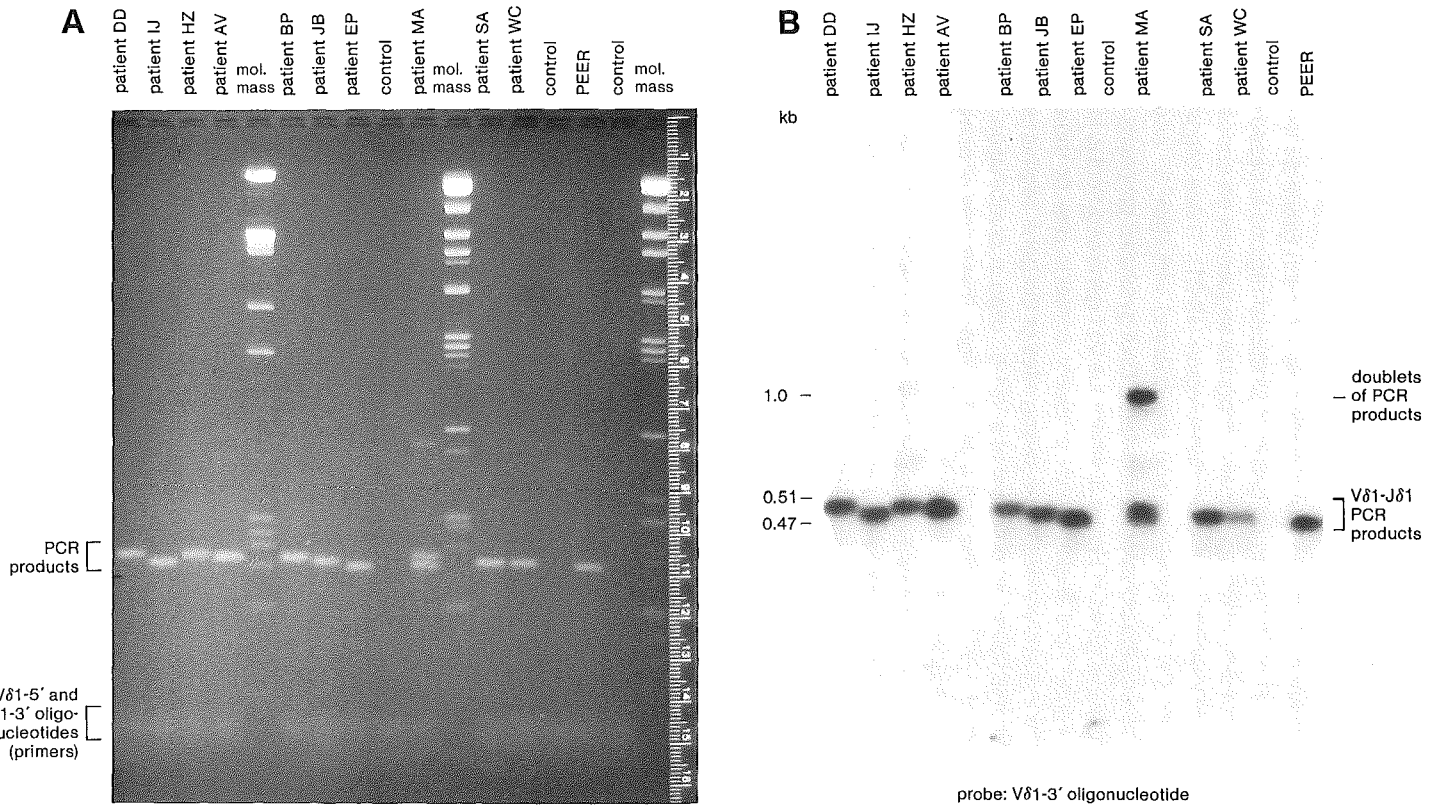


Figure 4. PCR analysis of the Vδ1-Jδ1 gene rearrangement in ten TcR-γδ$^+$ T-ALL. The cell line PEER was used as a positive control. PCR-mediated amplification of the junctional regions was performed by the use of the Vδ1-5′ and the Jδ1-3′ oligonucleotides as primers (see Figure 3 for oligonucleotides). The PCR products were separated in an ethidium bromide-stained agarose gel (**A**) and blotted to a nylon membrane which was hybridized with the ^{32}P-labeled Vδ1-3′ oligonucleotide (**B**). The results indicate that the size of the PCR products vary from approximately 470 to 510 bases, which implies that the junctional regions can vary extensively (~40 bases). The weak 1.0-kb band in the lane of patient HZ and the strong 1.0-kb band in the lane of patient MA represent doublets of PCR products. The origin of these doublets may be related to the fact that the leukemic cells of these two patients had Vδ1-Jδ1 gene rearrangements on both alleles.

PCR-mediated TcR-γ and TcR-δ gene analysis for detection of minimal residual disease in precursor B-ALL

The frequency of intra-lineage and cross-lineage Ig and TcR gene rearrangements in precursor B-ALL and T-ALL are summarized in Table 1 (see Chapter 6.2 for details). Based on these data the frequencies of Ig and TcR gene rearrangements in the total group of ALL has been calculated, assuming that precursor B-ALL account for ~80% of ALL, while T-ALL account for the remaining ~20%. Table 1 indicates that IgH, TcR-γ and TcR-δ gene rearrangements occur in high frequency in precursor B-ALL as well as in the total group of ALL. Several recent publications suggest that the PCR-mediated amplification of leukemia-specific junctional regions can be used in case of rearranged IgH, TcR-γ and TcR-δ genes (15-17). It should be emphasized that oligoclonality at diagnosis (22-28) and clonal evolution at relapse (29-31) as detected by IgH gene rearrangement studies, will severely hamper the application of this technique in precursor B-ALL, because changes in IgH gene rearrangements and subclone formation will lead to false negative results (Chapter 6.3). However, cross-lineage TcR-γ and TcR-δ gene rearrangements occur in high frequency in precursor B-ALL (Table 1) and it might well be that subclone formation at the IgH gene level is not paralleled by changes in the cross-lineage TcR gene rearrangement patterns. If so, the PCR-mediated amplification of TcR-γ and TcR-δ junctional regions will be an reliable procedure for the detection of minimal residual disease in precursor B-ALL with cross-lineage TcR gene rearrangements.

An advantage in the PCR-mediated analysis of TcR-γ and TcR-δ genes is that the combinatorial repertoire of these genes is low (32-37), while the junctional repertoire is high (35,38-40). This implies that only limited numbers of oligonucleotide primers are necessary, while the leukemia-specific junctional region probes will differ extensively between the leukemias. The latter enhances the specificity of the technique.

The extensive differences in the junctional regions of rearranged TcR-δ genes is illustrated by the results of our studies on Vδ and Jδ gene usage in ten TcR-γδ$^+$ T-ALL (Figure 4) (Breit et al., unpublished results). It appeared that all ten leukemias had a Vδ1-Jδ1 gene rearrange-

TABLE 1. Ig and TcR gene rearrangements in the total group of ALL.

	IgH	Igκ	Igλ	TcR-β	TcR-γ	TcR-δ	
	R[a]	R or D[a]	R	R	R	R	D
Precursor B-ALL[b]	98%	32%	6%	33%	55%	54%	26%
(~80% of ALL)	n=251	n=159	n=115	n=318	n=289	n=97	
T-ALL[c]	14%	0%	0%	89%	91%	68%	28%
(~20% of ALL)	n=180	n=62	n=59	n=281	n=209	n=116	
TOTAL[d]	81%	26%	5%	44%	62%	57%	26%

a. Symbols: R, one or both alleles rearranged; D, both alleles deleted.
b. The data concerning Ig and TcR gene rearrangements in precursor B-ALL are derived from Tables 2 and 5 in Chapter 6.2.
c. The data concerning Ig and TcR gene rearrangements in T-ALL are derived from Tables 3 and 4 in Chapter 6.2.
d. Calculated percentages, based on the assumption that ~80% of ALL represent precursor B-ALL, while ~20% represent T-ALL.

ment on at least one allele and in two cases on both alleles (patients HZ and MA). PCR-mediated amplification of the junctional regions was performed by use of the Vδ1-5′ and Jδ1-3′ oligonucleotides as primers (see Figure 3 for oligonucleotides). The PCR products were separated in an agarose gel and blotted to a nylon membrane, which was hybridized with the ^{32}P-labeled Vδ1-3′ oligonucleotide. The results indicate that the size of the PCR products vary from approximately 470 to 510 bases, which indicates that the size of the junctional region can vary extensively (~40 bases). Recent publications (41,42) and our own experience (Breit et al., unpublished results) indicate that Vδ1-Jδ1 gene rearrangements occur frequently in T-ALL, while Vδ2-Dδ3 and Dδ2-Dδ3 rearrangements are frequently observed in precursor B-ALL.

Theoretically, the detection limit of the PCR technique is approximately 0.001 to 0.0001%, i.e. one malignant cell between 10^5 to 10^6 normal cells. This is based on the calculation that a PCR tube can maximally contain 1 to 10 μg of genomic DNA, while one human cell contains approximately 10 pg of DNA. It should be noted that normal (polyclonal) T and B cells may have rearranged the same V to the same J gene segment as the leukemic cells. It is not known to what extent this normal background complicates the analysis of the PCR-mediated amplification of leukemia-specific junctional regions for the detection of minimal residual disease. For instance, normal TcR-γδ$^+$ T lymphocytes which use Vδ1 in combination with Jδ1, occur in PB in a frequency of 0.07-0.7% (Figure 5), but the junctional regions of these normal Vδ1-Jδ1$^+$ T cells probably differ extensively from each other as well as from the junctional regions of the leukemic cells. This emphasizes the necessity to produce leukemia-specific junctional region probes. Future studies will establish the real detection limit of the proposed PCR analyses and will reveal whether the background of normal cells which use the same V and J gene segments as the leukemic cells, may cause false positive results.

Of course not only precursor B-ALL patients, but also T-ALL patients may benefit from the PCR-mediated analysis of TcR-γ and/or TcR-δ genes for the detection of minimal residual disease. However, in case of T-ALL the above described T cell marker/TdT double IF staining is as yet a more established technique, about which already much information concerning sensitivity and specificity is available.

Chromosome aberrations and the detection of minimal residual disease

In the initial studies on the use of the PCR technique for the detection of minimal residual disease, chromosome aberrations such as t(14;18)(q32;q21) and t(9;22)(q34;q11) were used as tumor-specific markers (44-46). Oligonucleotide primers have been designed to recognize sequences at opposite sides of the breakpoint recombination area, so that the PCR product represents the tumor-specific hybrid DNA segment. However, only ~10% of ALL have a detectable specific chromosome translocation such as t(4;11)(q21;q23), t(1;19)(q23;p13.3), t(8;14)(q24;q32), t(11;14)(p13;q11) or t(9;22)(q34;q11) (47-49).

Another interesting approach for the detection of minimal disease in malignancies with a specific chromosome translocation, is the use of antibodies against the possible protein product of the fusion gene, i.e. the new hybrid gene which is caused by the translocation. For instance, the *bcr-abl* protein product, which can be produced in leukemias with t(9;22)(q34;q11) is a useful leukemia-specific marker (50). The sensitivity of such antibody techniques is probably comparable to that of the T cell marker/TdT double IF staining.

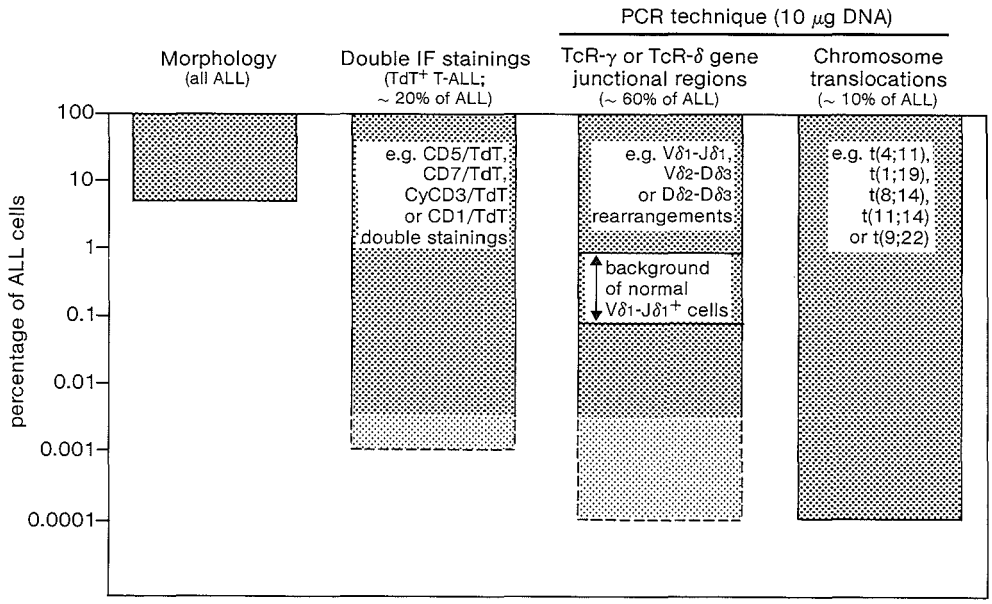


Figure 5. Detection limits of several techniques which can be used for the detection of minimal residual disease in ALL patients. Morphological techniques: ~5%. Double IF staining techniques for a T cell marker and TdT: 0.01-0.001%. PCR-mediated amplification of TcR-γ and TcR-δ junctional regions: 0.01 to 0.0001% (it is not yet known to what extent this detection limit is influenced by the "background" of normal cells which use the same V and J gene segments as the leukemic cells). PCR-mediated amplification of translocation-associated breakpoint recombination areas: 0.0001%. The frequencies of ALL for which the mentioned techniques can be used, are indicated in parentheses.

CONCLUSION

To our experience, immunologic marker analysis and analysis of Ig and TcR genes represent important tools in the diagnostics of leukemias and NHL. The combined morphological, immunophenotypic and genotypic information results in a more accurate diagnosis in patients with lymphoproliferative diseases or related disorders. This is important for the optimal choice of therapeutic regimens.

Development and clinical evaluation of techniques for the detection of low numbers of malignant cells in ALL patients is another major effort of our laboratory. The above discussed data indicate that no uniform technique for the detection of minimal residual disease in ALL exists, but that the technique of choice is determined by the immunophenotype, Ig/TcR genotype or the presence of a chromosome translocation, as indicated in Figure 5. This figure also summarizes the detection limits of the various techniques. It is obvious that a diagnostic laboratory should possess both detailed background information and broad experience in several fields in order to be able to choose the appropriate technique for the detection of minimal residual disease in each ALL patient. This is the more true since the application of either of these techniques is relatively expensive.

Highly specific and sensitive techniques for the detection of minimal residual disease enable adjustment of remission and relapse criteria as well as individualization of therapy, which allows prevention of undertreatment and overtreatment of the patients involved. Whether such adaptations of treatment protocols will result in lower relapse rates and higher survival rates should be evaluated in prospective studies.

REFERENCES

1. Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: M Nijhoff Publishers, 1984.
2. Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: M Nijhoff Publishers, 1986.
3. Bradstock KF, Papageorgiou ES, Janossy G, Hoffbrand AV, Willoughby ML, Roberts PD, Bollum FJ. Detection of leukaemic lymphoblasts in CSF by immunofluorescence for terminal transferase. Lancet 1980;i:1144.
4. Peiper S, Stass SA, Bollum FJ. Detection of leukaemic lymphoblasts in CSF. Lancet 1980;ii:39.
5. Hooijkaas H, Van Dongen JJM, Hählen K, Van Zanen GE. Immunological characterisation of cells in cerebrospinal fluid from patients with lymphoid malignancies. Lancet 1984;i:518-9.
6. Janossy G, Tidman N, Papageorgiou ES, Kung PC, Goldstein G. Distribution of T lymphocyte subsets in the human bone marrow and thymus: an analysis with monoclonal antibodies. J Immunol 1981;126:1608-13.
7. Neudorf SML, Le Bien TW, Bollum F, Kersey JH. Double fluorochrome analysis of human bone marrow lymphoid cells: studies with terminal transferase and anti-T cell monoclonal antibodies. Exp Hematol 1984;12:69-73.
8. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: M Nijhoff Publishers, 1984;67-81.
9. Bradstock KF, Kerr A, Bollum FJ. Antigenic phenotype of TdT-positive cells in human peripheral blood. Cell Immunol 1985;90:590-8.
10. Campana D, Thompson JS, Amlot P, Brown S, Janossy G. The cytoplasmic expression of CD3 antigens in normal and malignant cells of the T lymphoid lineage. J Immunol 1987;138:648-55.
11. Smith RG, Kitchens RL. Phenotypic heterogeneity of TdT^+ cells in the blood and bone marrow: implications for surveillance of residual leukemia. Blood 1989;74:312-9.
12. Bradstock KF, Janossy G, Tidman N, Papageorgiou ES, Prentice HG, Willoughby M, Hoffbrand AV. Immunological monitoring of residual disease in treated thymic acute lymphoblastic leukaemia. Leuk Res 1981;5:301-9.
13. Bradstock KF, Kerr A. Immunological detection of overt leukaemic spread in mediastinal T-cell lymphoblastic lymphoma. Leuk Res 1985;9:905-11.
14. Weinstein HJ, Vance ZB, Jaffe N, Buell D, Cassady JR, Nathan DG. Improved prognosis for patients with mediastinal lymphoblastic lymphoma. Blood 1979;53:687-94.
15. D'Auriol L, Macintyre E, Galibert F, Sigaux F. In vitro amplification of T cell γ gene rearrangements: a new tool for the assessment of minimal residual disease in acute lymphoblastic leukemias. Leukemia 1989;3:155-8.
16. Yamada M, Hudson S, Tournay O, Bittenbender S, Shane SS, Lange B, Tsujimoto Y, Caton AJ, Rovera G. Detection of minimal disease in hematopoietic malignancies of the B-cell lineage by using third-complementary-determining region (CDR-III)-specific probes. Proc Natl Acad Sci USA 1989;86:5123-7.
17. Hansen-Hagge TE, Yokota S, Bartram CR. Detection of minimal residual disease in acute lymphoblastic leukemia by in vitro amplification of rearranged T-cell receptor δ chain sequences. Blood 1989;74:1762-7.
18. Erlich HA, Gelfand DH, Saiki RK. Specific DNA amplification. Nature 1988;331:461-2.
19. White TJ, Arnheim N, Erlich HA. The polymerase chain reaction. Trends Genet 1989;5:185-9.
20. Bell J. The polymerase chain reaction. Immunol Today 1989;10:351-5.
21. Eisenstein BI. The polymerase chain reaction: a new method of using molecular genetics for medical

diagnosis. N Engl J Med 1990;322:178-83.
22. Foa R, Migone N, Saitta M, Fierro MT, Giubellino MC, Lusso P, Cordero di Montezemolo L, Miniero R, Lauria F. Different stages of B cell differentiation in non-T acute lymphoblastic leukemia. J Clin Invest 1984;74:1756-63.
23. Kitchingman GR, Mirro J, Stass S, Rovigatti U, Melvin SL, Williams DL, Raimondi SC, Murphy SB. Biologic and prognostic significance of the presence of more than two μ heavy-chain genes in childhood acute lymphoblastic leukemia of B precursor cell origin. Blood 1986;67:698-703.
24. Raghavachar A, Bartram CR, Ganser A, Heil G, Kleihauer E, Kubanek B. Acute undifferentiated leukemia: implications for cellular origin and clonality suggested by analysis of surface markers and immunoglobulin gene rearrangement. Blood 1986;68:658-62.
25. Rechavi G, Brok-Simoni F, Katzir N, Mandel M, Umiel T, Stark B, Zaizov R, Ben-Bassat I, Ramot B. More than two immunoglobulin heavy chain J region genes in the majority of infant leukemia. Leukemia 1988;2:347-50.
26. Bird J, Galili N, Link M, Stites D, Sklar J. Continuing rearrangement but absence of somatic hypermutation in immunoglobulin genes of human B cell precursor leukemia. J Exp Med 1988;168:229-45.
27. Ludwig W-D, Bartram CR, Harbott J, Köller U, Haas OA, Hansen-Hagge T, Heil G, Seibt-Jung H, Teichmann JV, Ritter J, Knapp W, Gardner H, Thiel E, Riehm H. Phenotypic and genotypic heterogeneity in infant acute leukemia. I. Acute lymphoblastic leukemia. Leukemia 1989;3:431-9.
28. Katz F, Ball L, Gibbons B, Chessells J. The use of DNA probes to monitor minimal residual disease in childhood acute lymphoblastic leukaemia. Br J Haematol 1989;73:173-80.
29. Wright JJ, Poplack DG, Bakhshi A, Reaman G, Cole D, Jensen JP, Korsmeyer SJ. Gene rearrangements as markers of clonal variation and minimal residual disease in acute lymphoblastic leukemia. J Clin Oncol 1987;5:735-41.
30. Raghavachar A, Thiel E, Bartram CR. Analyses of phenotype and genotype in acute lymphoblastic leukemias at first presentation and in relapse. Blood 1987;70:1079-83.
31. Raghavachar A, Ludwig W-D, Bartram CR. Clonal variation in childhood acute lymphoblastic leukaemia at early and late relapse detected by analyses of phenotype and genotype. Eur J Pediatr 1988;147:503-7.
32. LeFranc M-P, Forster A, Baer R, Stinson MA, Rabbitts TH. Diversity and rearrangement of the human T cell rearranging γ genes: nine germline variable genes belonging to two subgroups. Cell 1986;45:237-46.
33. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T cell γ chain joining regions and T cell development. J Immunol 1987;138:2687-90.
34. Lefranc M-P, Rabbitts TH. The human T-cell receptor γ (TRG) genes. Trends Biochem Sciences 1989;14:214-8.
35. Loh EY, Cwirla S, Serafini AT, Phillips JH, Lanier LL. The human T-cell receptor delta chain: genomic organization, diversity, and expression in populations of cells. Proc Natl Acad Sci USA 1988;85:9714-8.
36. Hata S, Clabby M, Devlin P, Spits H, De Vries JE, Krangel MS. Diversity and organization of human T cell receptor δ variable gene segments. J Exp Med 1989;169:41-57.
37. Takihara Y, Reimann J, Michalopoulos E, Ciccone E, Moretta L, Mak TW. Diversity and structure of human T cell receptor δ chain genes in peripheral blood γ/δ-bearing T lymphocytes. J Exp Med 1989;169:393-405.
38. Quertermous T, Strauss W, Murre C, Dialynas DP, Strominger JL, Seidman JG. Human T-cell γ genes contain *N* segments and have marked junctional variability. Nature 1986;322:184-7.
39. Hata S, Satyanarayana K, Devlin P, Band H, McLean J, Strominger JL, Brenner MB, Krangel MS. Extensive junctional diversity of rearranged human T cell receptor δ genes. Science 1988;240:1541-4.
40. Koning F, Knot M, Wassenaar F, Van den Elsen P. Phenotypical heterogeneity among human T cell receptor γ/δ-expressing clones derived from peripheral blood. Eur J Immunol 1989;19:2099-105.
41. Macintyre E, D'Auriol L, Amesland F, Loiseau P, Chen Z, Boumsell L, Galibert, Sigaux F. Analysis of junctional diversity in the preferential Vδ1-Jδ1 rearrangement of fresh T-acute lymphoblastic leukemia cells by in vitro gene amplification and direct sequencing. Blood 1989;74:2053-61.
42. Biondi A, Di Celle PF, Rossi V, Casorati G, Matullo G, Giudici G, Foa R, Migone N. High prevalence of T-cell receptor Vδ2-(D)-Dδ3 or Dδ1/2-Dδ3 rearrangements in B-precursor acute lymphoblastic leukemias. Blood 1990;75:1834-40.
43. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from

human peripheral blood. Eur J Immunol 1989;19:1559-68.
44. Lee M-S, Chang K-S, Cabanillas F, Freireich EJ, Trujillo JM, Stass SA. Detection of minimal residual cells carrying the t(14;18) by DNA sequence amplification. Science 1987;237:175-8.
45. Crescenzi M, Seto M, Herzig GP, Weiss PD, Griffith RC, Korsmeyer SJ. Thermostable DNA polymerase chain amplification of t(14;18) chromosome breakpoints and detection of minimal residual disease. Proc Natl Acad Sci USA 1988;85:4869-73.
46. Morgan GJ, Hughes T, Janssen JWG, Gow J, Guo A-P, Goldman JM, Wiedemann LM, Bartram CR. Polymerase chain reaction for detection of residual leukaemia. Lancet 1989;i:928-9.
47. Williams DL, Look AT, Melvin SL, Roberson PK, Dahl G, Flake T, Stass S. New chromosomal translocations correlate with specific immunophenotypes of childhood acute lymphoblastic leukemia. Cell 1984;36:101-9.
48. Ahuja HG, Cline MJ. Review: genetic and cytogenetic changes in acute lymphoblastic leukemia. Med Oncol Tumor Pharmacother 1988;5:211-22.
49. Uckun FM, Gajl-Peczalska KJ, Provisor AJ, Heerema NA. Immunophenotype-karyotype associations in human acute lymphoblastic leukemia. Blood 1989;73:271-80.
50. Van Denderen J, Hermans A, Meeuwsen T, Troelstra C, Zegers N, Boersma W, Grosveld G, Van Ewijk W. Antibody recognition of the tumor-specific *bcr-abl* joining region in chronic myeloid leukemia. J Exp Med 1989;169:87-98.

SUMMARY

T lymphocytes play a central role in the regulation of antigen-specific immune responses. The antigen-specificity of most T cell functions is achieved via the T cell receptor (TcR), which represents the receptor for specific recognition of foreign antigens, a process which is dependent on antigen-presentation via major histocompatibility complex molecules.

The TcR is closely associated with the CD3 protein chains which together form the TcR-CD3 complex on the cell surface of T lymphocytes. In this TcR-CD3 complex the CD3 protein chains probably play a role in the signal transduction from the TcR to the cytoplasm. Two types of TcR have been identified: the classical TcR, which consists of a TcR-α and a TcR-β chain, and the alternative TcR, consisting of a TcR-γ and a TcR-δ chain. The four TcR chains are encoded by genes which contain variable numbers of different V, (D) and J gene segments. During T cell differentiation these TcR genes have to rearrange before they can be transcribed and translated into functional protein products (Chapter 3.2).

The aim of this thesis was to study the various aspects of human T cell differentiation, especially the mechanisms which determine the expression of the TcR-CD3 complex, and to study how this information about T cell differentiation can be used for the development of new diagnostic tools for hematopoietic malignancies and immunodeficiencies. In these studies we have used peripheral blood, bone marrow and thymus samples from individuals without hematologic, immunologic or infectious disease. In addition, we used cell samples from patients with DiGeorge anomaly (DGA), a primary T cell immunodeficiency and from patients with a T cell malignancy. Especially T cell acute lymphoblastic leukemias (T-ALL) have been used in our studies, because these malignancies are generally regarded as clonally-expanded malignant counterparts of cells in the various normal differentiation stages.

Studying T cell differentiation by use of normal cell samples implies that the various cell types have to be studied at the single cell level, because normal cell samples are heterogeneous. For optimal immunophenotypic studies of such heterogeneous cell samples we developed several double and triple immunologic staining techniques, which allowed us to obtain detailed information about the expression of immunologic markers by small T cell populations. Detailed information about immunologic markers and the various techniques for marker analysis are described in Chapter 2.

For genotypic studies, i.e. analysis of rearrangement and transcription of TcR genes, especially T-ALL have been used, because the clonal origin of the leukemic cells makes complex cell separations and purifications superfluous. For optimal analysis of the TcR genes in the various leukemic cell samples we collected the relevant TcR probes and firstly studied the germline configuration of the TcR-β, TcR-γ and TcR-δ genes. Detailed information about the techniques used, the various TcR probes and the germline configuration of the TcR genes is given in Chapter 3.2 as well as in Chapters 4.5 and 4.7.

The results of our studies on T cell differentiation are extensively described in Chapter 4. They suggest that the precursors of T cells in the bone marrow, the so-called prothymocytes, express the pan-T cell markers CD2 and CD7 and that they are positive for HLA-DR and the

enzyme terminal deoxynucleotidyl transferase (TdT) (Chapter 4.2). TdT plays an important role in the generation of diversity in TcR molecules, based on its capability to insert nucleotides at the joining sites of V, D and J gene segments during rearrangement of the TcR genes. These TcR gene rearrangement processes probably start as soon as the immature T cells have arrived in the thymus and seem to occur in a hierarchic order. The results of the TcR gene studies in T-ALL suggest that the TcR-δ genes rearrange firstly, immediately followed by rearrangement of the TcR-γ genes and in most cells also by rearrangement of the TcR-β genes. Probably the TcR-γ gene rearrangements firstly involve the γ1 locus and if this rearrangement is non-functional, rearrangement to the γ2 locus occurs. Transcription of functional TcR-γ and TcR-δ genes results in TcR-$\gamma\delta^+$ T cells. If the TcR-γ and/or TcR-δ gene rearrangements are not functional or incomplete, the involved T cells may be eliminated. Alternatively these T cells or other immature T cells may delete one or both TcR-δ genes and rearrange their TcR-β and TcR-α genes. In T cells with rearranged TcR-α genes the TcR-δ genes have been deleted, because the TcR-δ gene locus is located within the TcR-α gene locus, between the Vα and Jα gene segments. The TcR-δ gene deletion and TcR-α gene rearrangement most probably represent two consecutive steps. Functional rearrangement of the TcR-α and TcR-β genes results in TcR-$\alpha\beta^+$ T cells (Chapters 4.3, 4.4, 4.5 and 4.7). In immature T cells, which do not (yet) express the TcR-CD3 complex on their cell membrane, the CD3 genes are already transcribed and translated into CD3 protein chains, which accumulate in the cytoplasm, ready for assemblage into TcR-CD3 complexes as soon as the appropriate TcR chains are produced (Chapters 4.3 and 5.2).

As indicated above, the T-ALL data suggest that TcR-$\gamma\delta^+$ and TcR-$\alpha\beta^+$ cells are derived from a linear differentiation pathway. However, recent data from studies in mice suggest that TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ cells develop along separate differentiation lineages and that several regulatory mechanisms involving the TcR-α/δ locus play a central role in the early divergence of the $\alpha\beta$ and $\gamma\delta$ pathways (reviewed in Chapter 4.1).

In man the frequency of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ cells can be determined by use of monoclonal antibodies against non-polymorphic epitopes of the two types of TcR (Chapter 4.6). To study the thymus-dependency of TcR-$\alpha\beta^+$ and TcR-$\gamma\delta^+$ T lymphocytes, we have enumerated these cells in blood samples from healthy children as well as from eight infants with DGA. DGA is characterized by facial, cardiac, parathyroid and thymic defects. The degree of thymus hypoplasia or aplasia in DGA is assumed to determine the degree of T cell immunodeficiency (i.e. from partial to complete). In healthy children the majority of CD3$^+$ lymphocytes express TcR-$\alpha\beta$, while only a minority express TcR-$\gamma\delta$. In seven infants with partial DGA the variable degrees of decrease in T lymphocytes mainly involved the TcR-$\alpha\beta^+$ cells, whereas the absolute numbers of TcR-$\gamma\delta^+$ cells were much less affected. In one patient with complete DGA the great majority of the few remaining T lymphocytes expressed TcR-$\gamma\delta$, but virtually no TcR-$\alpha\beta^+$ T lymphocytes were found. These data indicate that the T cell deficiency in DGA patients is mainly restricted to TcR-$\alpha\beta^+$ T lymphocytes, which implies that the development of TcR-$\alpha\beta^+$ cells is severely hampered by the decrease in thymic mass in DGA, while the TcR-$\gamma\delta$ lineage is less affected. This suggests that TcR-$\gamma\delta^+$ T lymphocytes in man can develop along an extrathymic differentiation pathway, which is supported by the observation that TcR-$\gamma\delta^+$ cortical thymocytes and especially TcR-$\gamma\delta^+$/TdT$^+$ thymocytes are rare (Chapters 4.1 and 4.8).

The various techniques and the obtained knowledge on human T lymphocyte differentiation appeared to be useful for diagnostic purposes. For instance, the cytoplasmic expression of CD3 (CyCD3) appeared to be an excellent marker for immature T cell malignancies, which do not express a TcR-CD3 complex on the cell surface (Chapter 5.2). In addition, the experience concerning immunologic marker analysis of normal and malignant T cells could easily be extended to immunologic marker analysis of other hematopoietic cells and their malignant counterparts (Chapter 2.3). This allowed us to compose optimal panels of immunologic markers for immunophenotyping of the various types of leukemias and non-Hodgkin lymphomas (NHL). Immunophenotyping of leukemias and NHL in addition to routine cytomorphology is important for a detailed and reproducible classification of these malignancies. In ~30% of cases this will lead to a more appropriate or more accurate diagnosis than by use of cytomorphology alone, which is important to institute the appropriate therapy (Chapter 5.1).

The experience with the analysis of TcR genes was extended to the analysis of immunoglobulin (Ig) genes (Chapter 3.3). The great majority of lymphoid malignancies of B and T cell origin have clonally rearranged Ig and TcR genes, respectively. Also cross-lineage Ig and TcR gene rearrangements occur. This especially concerns TcR gene rearrangements in precursor B-ALL (30 to 80%, depending on the type of TcR gene), Ig heavy chain (IgH) gene rearrangements in T-ALL (5 to 15%) and Ig and/or TcR gene rearrangements in acute myeloid leukemia (AML) (~20% of all AML). Detailed information about these Ig and TcR gene rearrangements in hematopoietic malignancies is summarized in Chapter 6.2. This information can be used for diagnostic purposes, because Southern blot analysis of Ig and TcR genes is an excellent tool for the detection of clonality. This allows the following diagnostic applications: discrimination between polyclonal and monoclonal lymphoproliferations; detection of two or more subclones within a malignancy at diagnosis; detection of clonal evolution at relapse; analysis of the clonal origin of two lymphoid malignancies in a single patient; and analysis of the differentiation lineage of a malignancy. Although virtually all malignancies are clonal proliferations, it should be noticed that a clonal cell population does not always represent a malignancy (Chapter 6.3).

In the majority of patients with a lymphoproliferative disease morphological techniques and immunologic marker analysis give sufficient information, but in 1 to 5% of cases gene rearrangement studies are necessary to make a correct diagnosis. The application of Ig and TcR gene analysis for diagnostic purposes should be restricted to these cases, because the Southern blot technique is expensive and time-consuming. Much background information concerning the technical possibilities and limitations of Ig and TcR gene analysis as well as broad experience are needed for correct interpretation of the Southern blot data in conjunction with the results of immunologic marker analysis. This is especially true for clinically-benign lymphoproliferations, which are frequently seen in immunodeficiency patients (Chapter 6.3).

The detection of small numbers of malignant cells is a major problem at diagnosis and during follow-up of malignancies. One of the most frightening vexations for patients and their doctors in attendance is the uncertainty whether or not all malignant cells have been eradicated by the treatment protocols. This is especially the case in childhood ALL, because these leukemias may relapse up to five years or more after initial diagnosis and subsequent treatment. Therefore, we have put a lot of effort in designing techniques for detection of minimal (residual) disease in ALL. The detection limit of conventional morphological techniques is not

lower than 1 to 5% (i.e. 1 to 5 malignant cells between 100 normal cells) since small numbers of malignant cells are difficult to discriminate from normal cells. Also the application of immunologic marker analysis for the detection of low numbers of malignant cells is hampered by the presence of normal counterparts which express the same markers. However, in specific cases it is possible to detect minimal disease based on the assumption that the presence of positive cells outside their normal "homing areas" is indicative for malignancy (Chapter 5.3). In this way TdT can be used as marker for the detection of ALL cells in cerebrospinal fluid. This allows the early detection (or exclusion) of central nervous system leukemia, which is a severe complication in ALL patients (Chapter 5.4). Another example is the detection of extremely low numbers of malignant cells in bone marrow and blood of patients with a TdT^+ T cell malignancy by use of a double labeling technique for a T cell marker and TdT. This application is based on the observation that certain T cell $marker^+/TdT^+$ cells normally occur in the thymus only and not in extrathymic locations such as bone marrow and blood. Due to the low detection limit of the double staining technique (0.01 to 0.001%), it is possible to diagnose a relapse of a TdT^+ T cell malignancy 2 to 6 months earlier than by use of conventional cytomorphology (Chapter 5.3).

Immunologic marker analysis cannot efficiently be used for the detection of minimal residual disease in patients with precursor B-ALL, because normal counterparts of such leukemic cells occur in relatively high frequencies in bone marrow and blood, especially during the period of bone marrow regeneration after withdrawal of cytotoxic treatment. However, recent reports indicate that the polymerase chain reaction (PCR) technique may be useful for the detection of minimal residual disease via the amplification of the junctional regions of rearranged Ig and TcR genes. This is based on the fact that the junctional regions of rearranged Ig and TcR genes can vary enormously due to the differential ways of joining of V, (D) and J gene segments and the insertion of additional nucleotides at the joining sites (Chapter 3.2). The junctional regions probably differ in each lymphocyte (clone) and therefore also in each lymphoid leukemia. It has been suggested that the PCR-mediated amplification of leukemia-specific junctional regions can be used in case of rearranged IgH, TcR-γ and TcR-δ genes. The theoretical detection limit of the PCR technique is 0.01 to 0.0001%. Future studies will establish the real detection limit of the proposed PCR analyses and reveal the frequency of false negative and/or false positive results.

Highly specific and sensitive techniques for the detection of minimal residual disease allow adjustment of remission and relapse criteria as well as individualization of therapy, which is valuable for the prevention of undertreatment and overtreatment of patients. Whether such adaptations of treatment protocols will result in lower relapse rates and higher survival rates has to be evaluated in prospective studies.

SAMENVATTING

T-lymfocyten spelen een centrale rol in de regulatie van antigeen-specifieke immuunreacties. De antigeen-specificiteit van de meeste T-cel functies wordt gerealiseerd via de T-cel-receptor (TcR). Deze TcR is betrokken bij de specifieke herkenning van vreemde antigenen, een proces dat afhankelijk is van antigeenpresentatie via moleculen van het "major histocompatibility complex".

De TcR is nauw verbonden met de CD3 eiwitketens. Tezamen vormen zij het TcR-CD3 complex op het celoppervlak van de T-lymfocyten. Binnen dit TcR-CD3 complex spelen de CD3 eiwitketens waarschijnlijk een rol in de signaaloverdracht van de TcR naar het cytoplasma. Er bestaan twee soorten TcR: de "klassieke" TcR, die uit een TcR-α en een TcR-β keten bestaat, en de "alternatieve" TcR, welke gevormd wordt door een TcR-γ en een TcR-δ keten. De vier TcR ketens worden gecodeerd door genen die uit variabele aantallen verschillende V,(D) en J gensegmenten bestaan. Gedurende de differentiatie van T-cellen moeten deze TcR genen worden herschikt voordat door middel van transcriptie en translatie functionele eiwitproducten kunnen worden gevormd.

Het doel van dit promotie-onderzoek was enerzijds de verschillende aspecten van de T-cel differentiatie bij de mens te bestuderen, in het bijzonder de mechanismen die de expressie van het TcR-CD3 complex bepalen, en anderzijds te onderzoeken hoe basale informatie betreffende de T-cel differentiatie kan worden gebruikt voor het ontwikkelen van nieuwe diagnostische methoden voor hematopoiëtische maligniteiten en immunodeficiënties. In deze studies werden perifeer bloed-, beenmerg- en thymusmonsters van personen zonder hematologische, immunologische of infectieuze aandoeningen geanalyseerd. Bovendien werden celmonsters van zowel patiënten met het DiGeorge syndroom (DGS), een primaire T-cel immunodeficiëntie, als van patiënten met een T-cel maligniteit bestudeerd. Vooral T-cel acute lymfoblastaire leukemieën (T-ALL) werden in ons onderzoek gebruikt, aangezien deze maligniteiten gewoonlijk worden beschouwd als klonaal geëxpandeerde maligne tegenpolen van cellen in de verschillende normale differentiatiestadia.

Het gebruik van normale celmonsters bij het bestuderen van de T-cel differentiatie betekent dat de verschillende celtypen op "single cell" niveau moeten worden onderzocht, aangezien de celpopulaties van normale personen heterogeen van samenstelling zijn. Voor optimale immunofenotyperingstudies van dergelijke heterogene celmonsters ontwikkelden wij twee- en drievoudige immunologische kleuringstechnieken, waardoor het mogelijk was gedetailleerde informatie te verkrijgen over de expressie van immunologische markers door kleine populaties T-cellen. Gedetailleerde gegevens over immunologische markers en de verschillende technieken waarmee ze kunnen worden aangetoond staan beschreven in Hoofdstuk 2. Voor de analyse van de herschikking en transcriptie van de TcR genen werden vooral T-ALL cellen gebruikt, omdat de klonale oorsprong van de leukemiecellen complexe celscheidingen en celzuiveringen overbodig maakt. Om de TcR genen in de verschillende leukemische celmonsters optimaal te kunnen analyseren werden eerst de relevante TcR probes verzameld waarmee de kiemlijnconfiguratie van de TcR-β, de TcR-γ en de TcR-δ genen werd bestudeerd.

Gedetailleerde informatie over de gebruikte technieken, de verschillende gebruikte TcR probes en de kiemlijnconfiguratie van de TcR genen wordt gegeven in de Hoofdstukken 3.2, 4.5 en 4.7.

De resultaten van de T-cel differentiatiestudies staan uitgebreid beschreven in Hoofdstuk 4. Gevonden is, dat de voorloper T-cellen in het beenmerg, de zogenaamde prothymocyten, waarschijnlijk de pan-T-celmarkers CD2 en CD7 tot expressie brengen en dat zij positief zijn voor HLA-DR en het enzym terminaal deoxynucleotidyl transferase (TdT) (Hoofdstuk 4.2). Het enzym TdT speelt een belangrijke rol bij het ontstaan van de diversiteit in de TcR moleculen. Dit is gebaseerd op het feit dat TdT in staat is extra nucleotiden in te voegen op de zg. "joining sites" van V, D en J gensegmenten tijdens de herschikking van de TcR genen. Deze TcR genherschikkingsprocessen beginnen waarschijnlijk zodra de onrijpe T-cellen in de thymus zijn gearriveerd. Deze processen lijken in een hiërarchisch bepaalde volgorde plaats te vinden. De resultaten die werden verkregen bij het bestuderen van de TcR genen in T-ALL doen vermoeden dat eerst de TcR-δ genen herschikken, onmiddellijk gevolgd door de herschikking van de TcR-γ genen, en in de meeste cellen tevens door die van de TcR-β genen. Waarschijnlijk is bij de TcR-γ genherschikking eerst het γ1 locus betrokken en als deze γ1 herschikking niet-functioneel is, vindt herschikking naar het γ2 locus plaats. Transcriptie van functionele TcR-γ en TcR-δ genen resulteert in TcR-$\gamma\delta^+$ T-cellen. Indien de TcR-γ en/of TcR-δ genherschikkingen niet-functioneel of incompleet zijn, worden de betrokken T-cellen waarschijnlijk geëlimineerd. Het is ook mogelijk dat deze T-cellen of andere onrijpe T-cellen één of beide TcR-δ genen deleteren en hun TcR-β en TcR-α genen gaan herschikken. T-cellen met herschikte TcR-α genen hebben hun TcR-δ genen gedeleteerd, omdat het TcR-δ genlocus midden in het TcR-α genlocus ligt en wel tussen de Vα en Jα gensegmenten. De TcR-δ gendeletie en de TcR-α genherschikking zijn zeer waarschijnlijk twee opeenvolgende stappen in het TcR genherschikkingsproces. Functionele herschikking van de TcR-α en de TcR-β genen resulteert in TcR-$\alpha\beta^+$ T-cellen (Hoofdstukken 4.3, 4.4, 4.5 en 4.7). In onrijpe T-cellen, die (nog) geen TcR-CD3 complex op hun celmembraan tot expressie brengen, vindt al transcriptie en translatie van CD3 genen tot CD3 eiwitketens plaats. Deze ketens accumuleren in het cytoplasma, gereed voor assemblage tot TcR-CD3 complexen zodra de correcte TcR ketens worden geproduceerd (Hoofdstukken 4.3 en 5.2).

Zoals hiervoor al is aangegeven, doen de gegevens verkregen uit de analyse van T-ALL vermoeden dat TcR-$\gamma\delta^+$ en TcR-$\alpha\beta^+$ cellen uit een lineaire differentiatieroute afkomstig zijn. Recente gegevens afkomstig uit onderzoek bij de muis suggereren echter dat TcR-$\alpha\beta^+$ en TcR-$\gamma\delta^+$ cellen zich langs gescheiden differentiatielijnen ontwikkelen en dat verschillende regulatiemechanismen in het TcR-α/δ locus een centrale rol spelen in de vroege splitsing van de $\alpha\beta$ en $\gamma\delta$ differentiatieroutes (bediscussiëerd in Hoofdstuk 4.1).

Bij de mens kan de frequentie van TcR-$\alpha\beta^+$ en TcR-$\gamma\delta^+$ cellen worden bepaald met behulp van monoklonale antistoffen tegen de niet-polymorfe epitopen van de twee typen TcR (Hoofdstuk 4.6). Om de thymus-afhankelijkheid van TcR-$\alpha\beta^+$ en TcR-$\gamma\delta^+$ T-lymfocyten te bestuderen, hebben we hun frequentie bepaald in bloedmonsters afkomstig van normale kinderen en van acht kinderen met DGS. Het DGS wordt gekarakteriseerd door defekten aan gelaat, hart, bijschildklier en thymus. De mate van thymushypoplasie of thymusaplasie bepaalt waarschijnlijk de mate van T-cel immunodeficiëntie (d.w.z. van partieel tot volledig). Onze resultaten bij gezonde kinderen geven aan dat de meerderheid van de CD3$^+$ lymfocyten de TcR-$\alpha\beta$ tot expressie brengt en slechts een minderheid de TcR-$\gamma\delta$. Bij zeven kinderen met een

partieel DGS werd een wisselende vermindering van het aantal T-lymfocyten waargenomen. Dit betrof voornamelijk TcR-$\alpha\beta^+$ cellen, terwijl de absolute aantallen TcR-$\gamma\delta^+$ cellen slechts in geringe mate waren afgenomen. In één patiënt met een compleet DGS bracht het merendeel van het geringe aantal resterende T-lymfocyten de TcR-$\gamma\delta$ tot expressie en werden er vrijwel geen TcR-$\alpha\beta^+$ T-lymfocyten waargenomen. Dit betekent dat de T-cel deficiëntie in patiënten met een DGS zich voornamelijk beperkt tot de TcR-$\alpha\beta^+$ T-lymfocyten, hetgeen doet vermoeden dat de ontwikkeling van TcR-$\alpha\beta^+$ cellen ernstig wordt belemmerd door de afname van de thymusmassa bij het DGS, terwijl de TcR-$\gamma\delta$ differentiatielijn minder is aangetast. Dit suggereert dat bij de mens de TcR-$\gamma\delta^+$ T-lymfocyten zich langs een extrathymale differentiatieroute kunnen ontwikkelen. Dit wordt ondersteund door de bevinding dat TcR-$\gamma\delta^+$ corticale thymocyten en met name TcR-$\gamma\delta^+$/TdT$^+$ thymocyten zeldzaam zijn (Hoofdstukken 4.1 en 4.8).

De verschillende gebruikte technieken en de opgedane kennis omtrent T-cel differentiatie bij de mens bleken bruikbaar te zijn voor diagnostische toepassingen. Zo bleek de cytoplasmatische expressie van CD3 (CyCD3) een uitstekende marker te zijn voor onrijpe T-cel maligniteiten die geen TcR-CD3 complex op het celoppervlak tot expressie brengen (Hoofdstuk 5.2). Bovendien kon de ervaring, opgedaan bij de immunologische markeranalyse van normale en maligne T-cellen, worden benut voor de immunologische markeranalyse van andere hematopoiëtische cellen en hun maligne tegenpolen (Hoofdstuk 2.3). Dit stelde ons in staat optimale panels van immunologische markers samen te stellen voor de immunofenotypering van de verschillende typen leukemieën en non-Hodgkin lymfomen (NHL). De immunofenotypering van leukemieën en NHL is, naast de routinematige cytomorfologische analyse, van belang voor een gedetailleerde en reproduceerbare klassificatie van deze maligniteiten. In ~30% van de gevallen zal dit tot een meer correcte of meer nauwkeurige diagnose leiden dan wanneer alleen cytomorfologische technieken worden toegepast (Hoofdstuk 5.1).

De ervaring die was opgedaan met het analyseren van TcR genen werd ook benut voor het analyseren van de immunoglobuline (Ig) genen (Hoofdstuk 3.3). De grote meerderheid van lymfatische maligniteiten van B- en T-cel oorsprong hebben klonaal herschikte Ig genen, respectievelijk TcR genen. Ook kunnen "cross-lineage" Ig en TcR genherschikkingen optreden. Dit betreft met name TcR genherschikkingen in voorloper B-ALL (30 tot 80%, afhankelijk van het type TcR gen), Ig zware keten (IgH) genherschikkingen in T-ALL (5 tot 15%) en Ig en/of TcR genherschikkingen in acute myeloïde leukemieën (AML) (~20% van alle AML). Gedetailleerde informatie over deze Ig en TcR genherschikkingen in hematopoiëtische maligniteiten is samengevat in Hoofdstuk 6.2. Deze informatie kan voor diagnostische doeleinden worden gebruikt, omdat Southern blot analyse van Ig en TcR genen een uitstekende methode is om klonaliteit te detecteren. Dit maakt de volgende diagnostische toepassingen mogelijk: discriminatie tussen polyklonale en monoklonale lymfoproliferaties; detectie van twee of meer subklonen binnen één maligniteit; detectie van klonale evolutie bij een recidief; analyse van de klonale oorsprong van twee maligniteiten in één patiënt; en analyse van de differentiatielijn van een maligniteit. Alhoewel vrijwel alle maligniteiten klonale proliferaties zijn, moet opgemerkt worden dat een klonale celpopulatie niet altijd maligne is (Hoofdstuk 6.3).

In de meeste patiënten met een klonale lymfoproliferatieve aandoening geven morfologische technieken en immunologische markeranalyse voldoende informatie. Echter in 1 tot 5% van de gevallen zijn genherschikkingstudies noodzakelijk om een correcte diagnose te stellen. De

toepassing van Ig en TcR genanalyse voor diagnostische doeleinden dient beperkt te worden tot deze gevallen, omdat de "Southern blot" techniek kostbaar en tijdrovend is. Veel achtergrondinformatie betreffende de technische mogelijkheden en beperkingen van de Ig en TcR genanalyse en brede ervaring zijn nodig voor een correcte interpretatie van de "Southern blot" gegevens. Dit is met name het geval bij klinisch benigne lymfoproliferaties, die frequent worden gezien bij patiënten met een immunodeficiëntie (Hoofdstuk 6.3).

De detectie van kleine aantallen maligne cellen is een groot probleem bij de diagnose en follow-up van patiënten met een maligniteit. Eén van de grootste problemen voor patiënten en hun behandelende artsen is de onzekerheid over het al dan niet geëlimineerd zijn van alle maligne cellen door het gebruikte behandelingsprotocol. Dit is met name het geval in ALL bij kinderen, omdat deze leukemieën tot vijf jaar of langer na de initiële diagnose en de daarop volgende behandeling kunnen recidiveren. Om deze reden hebben wij veel energie gestoken in de ontwikkeling van technieken voor de detectie van kleine aantallen maligne cellen bij ALL. De detectiegrens van conventionele morfologische technieken is niet lager dan 1 tot 5% (1 tot 5 maligne cellen tussen 100 normale cellen), aangezien kleine aantallen maligne cellen moeilijk te onderscheiden zijn van normale cellen. Ook de toepassing van immunologische markeranalyse voor de detectie van kleine aantallen maligne cellen wordt vaak beperkt door de aanwezigheid van normale tegenpolen, die dezelfde markers tot expressie brengen. Toch is het in sommige gevallen mogelijk om kleine aantallen maligne cellen te detecteren, gebaseerd op de aanname dat de aanwezigheid van positieve cellen buiten hun normale plaatsen van voorkomen op een maligniteit wijst (Hoofdstuk 5.3). Op deze manier kan TdT worden gebruikt als marker voor de detectie van ALL cellen in liquor cerebrospinalis. Dit maakt vroeg detecteren (of uitsluiten) van centraal zenuwstelsel leukemie, een ernstige complicatie bij patiënten met ALL, mogelijk (Hoofdstuk 5.4). Een ander voorbeeld is de detectie van zeer kleine aantallen maligne cellen in beenmerg en bloed van patiënten met een TdT^+ T-cel maligniteit met behulp van een dubbellabelingstechniek voor een T-cel marker en TdT. Deze toepassing is gebaseerd op de waarneming dat bepaalde T-cel $marker^+/TdT^+$ cellen normaal slechts voorkomen in de thymus en niet in extrathymale locaties zoals beenmerg en bloed. Dankzij de lage detectiegrens van de dubbellabelingstechniek (0,01 tot 0,001%) is het mogelijk om een recidief van een TdT^+ T-cel maligniteit 2 tot 6 maanden eerder te diagnostiseren dan bij gebruik van conventionele cytomorfologie (Hoofdstuk 5.3).

Immunologische markeranalyse kan niet goed worden gebruikt voor de detectie van kleine aantallen maligne cellen bij patiënten met een voorloper B-ALL, omdat de normale tegenpolen van zulke leukemische cellen in relatief hoge frequenties vóórkomen in beenmerg en bloed, met name tijdens de periode van beenmergregeneratie na het beëindigen van de cytostatische therapie. Recente bevindingen geven echter aan dat de polymerase ketting reactie (polymerase chain reaction; PCR) bruikbaar zou kunnen zijn voor de detectie van kleine aantallen maligne cellen door middel van amplificatie van de "junctional regions" van herschikte Ig en TcR genen.Dit berust op het feit dat de "junctional regions" van herschikte Ig en TcR genen sterk kunnen variëren als gevolg van het samenkomen van V, (D) en J gensegmenten en het toevoegen van extra nucleotiden op de verbindingsplaatsen van de gensegmenten (Hoofdstuk 3.2). Deze "junctional regions" variëren waarschijnlijk per lymfocyt (kloon) en daarom ook per leukemie. Waarschijnlijk kan de PCR-gemediëerde amplificatie van de leukemie-specifieke "junctional regions" gebruikt worden voor het opsporen van kleine aantallen leukemie cellen in

geval van herschikte IgH, TcR-γ en/of TcR-δ genen. De theoretische detectiegrens van de PCR techniek is 0,01 tot 0,0001%. Toekomstig onderzoek zal inzicht moeten geven in de werkelijke detectiegrens van de voorgestelde PCR analyses, en duidelijk moeten maken hoe frequent vals negatieve en/of vals positieve resultaten worden verkregen.

Uiterst specifieke en gevoelige technieken voor de detectie van kleine aantallen maligne cellen maken zowel aanpassing van remissie- en recidiefcriteria, als individualisering van de therapie mogelijk, hetgeen van belang is voor de preventie van onderbehandeling en overbehandeling van patiënten. Prospectief onderzoek zal moeten uitwijzen of mogelijke aanpassingen van de behandelingsprotocollen zullen resulteren in kleinere recidiefkansen en grotere overlevingskansen.

ABBREVIATIONS

3´UT	: 3´ untranslated
5´-NT	: 5´-nucleotidase
6-MP	: 6-mercaptopurine
ADCC	: antibody dependent cytotoxicity
AET	: 2-amino-ethylisothiouronium bromide
AIDS	: acquired immunodeficiency syndrome
AILD	: angioimmunoblastic lymphadenopathy with dysproteinemia
AIM	: activation inducer molecule
ALL	: acute lymphoblastic leukemia
AML	: acute myeloid leukemia
ANLL	: acute non-lymphoblastic leukemia
AP	: alkaline phosphatase
APC	: allophycocyanin
ARA-C	: cytosine arabinoside
AT	: ataxia telangiectasia
ATG	: anti-thymocyte globulin
ATLL	: adult T cell leukemia lymphoma
AUL	: acute undifferentiated leukemia
B-ALL	: B cell ALL
B-CLL	: B cell CLL
B-NHL	: B cell NHL
B-PLL	: B cell PLL
BAL	: bronchoalveolar lavage
BC	: blast crisis
BM	: bone marrow
BMT	: bone marrow transplantation
bp	: base pairs
BrdU	: 5´-bromo-2´-deoxyuridine
BSA	: bovine serum albumin
C	: constant
CALLA	: common ALL antigen
CB	: centroblastic lymphoma
CB-CC	: centroblastic-centrocytic lymphoma
CC	: centrocytic lymphoma
CD	: cluster of differentiation/cluster of designation
CDR	: complementarity determining region
CDw	: CD "workshop" (preliminary clustering)
CID	: combined immunodeficiency
CLL	: chronic lymphocytic leukemia
CML	: chronic myeloid leukemia
CNS	: central nervous system
ConA	: concanavalin A
CR	: complement receptor
CSF	: cerebrospinal fluid
CTLL	: cutaneous T cell leukemia lymphoma
CyCD3	: cytoplasmic expression of CD3 antigen
CyCD22	: cytoplasmic expression of CD22 antigen
CyIg	: cytoplasmic Ig
Cyμ	: cytoplasmic μ Ig heavy chain
D	: diversity
DAF	: decay accelerating factor
DGA	: DiGeorge anomaly
DNA	: deoxyribonucleic acid
DPP IV	: dipeptidylpeptidase IV
DTT	: dithiothreitol
EBV	: Epstein-Barr virus
FAB	: French American British cytomorphological classification of acute leukemias
FAL	: fucosyl-N-acetyllactosamine
FcγR	: Fc receptor for IgG
FcϵR	: Fc receptor for IgE
FcμR	: Fc receptor for IgM
FCS	: fetal calf serum
FITC	: fluorescein isothiocyanate
FLS	: forward light scatter
G6PD	: glucose-6-phosphate dehydrogenase
GMP	: granule membrane protein
GP or gp	: glycoprotein
GpA	: glycophorin A
h	: hinge
HCL	: hairy cell leukemia
HEV	: high endothelial venules
HIV	: human immunodeficiency virus
HNK	: human natural killer cell
HTLV-I	: human T cell leukemia virus I
HVP	: hypervariable (or length) polymorphism
ICAM	: intercellular adhesion molecule
Id	: idiotype
IEF	: isoelectric focussing
IF	: immunofluorescence
IFN	: interferon
Ig	: immunoglobulin
IgH	: Ig heavy chain
IgL	: Ig light chain
IGSS	: immunogold silver staining
IL-2	: interleukin 2
IPB	: immunoprecipitation buffer
J	: joining
kb	: kilobase
kDa	: kilo Dalton
Kde	: kappa deleting element
LAD	: leukocyte adhesion deficiency
LAS	: lymphadenopathy syndrome

LBL : lymphoblastic lymphoma
LCA : leukocyte common antigen
LeuCAM : leukocyte adhesion molecule
LFA : leukocyte function antigen
LGL : large granular lymphocyte
LN : lymph node
McAb : monoclonal antibody/antibodies
MCP : membrane cofactor protein
MF : mycosis fungoides
MHC : major histocompatibility complex
MIC : morphologic, immunologic and cytogenetic classification
MNC : mononuclear cells
mo : month(s)
mol.mass : molecular mass
MPO : myeloperoxidase
MRBC : mouse red blood cells
MS : multiple sclerosis
MTX : methotrexate
N : nucleotide
NCAM : neural cell adhesion molecule
NHL : non-Hodgkin lymphoma
NK cell : natural killer cell
NP-40 : Nonidet P40 (detergent)
OLB : oligonucleotide labeling buffer
p : protein
PB : peripheral blood
PBL : peripheral blood lymphocytes
PBS : phosphate buffered saline
PCR : polymerase chain reaction
PE : pleural exudate
PHA : phytohaemagglutinin
PI : phosphatidyl-inositol glycan
pI : isoelectric point
PLEVA : pityriasis lichenoides et varioliformis acuta
PLL : prolymphocytic leukemia
PLS : perpendicular light scatter
PMSF : phenylmethylsulfonyl fluoride (protease inhibitor)
PNH : paroxysmal nocturnal hemoglobinuria
PO : peroxidase
R : receptor
RA : rheumatoid arthritis
RBC : red blood cells
RFLP : restriction fragment length polymorphism
RNA : ribonucleic acid
RS cell : Reed-Sternberg cell
RT : room temperature
s : switch
SCID : severe combined immunodeficiency
SDS : sodium dodecyl sulfate
SDS-PAGE : sodium dodecyl sulfate polyacrylamide gel electrophorese
SITS : acetamide isothiocyanate stilbene disulphonic acid
SmCD3 : surface membrane CD3 antigen
SmIg : surface membrane Ig
SRBC : sheep red blood cells
SS : Sézary syndrome
SSPE : subacute sclerosing panencephalitis
T-ALL : T cell ALL
T-CLL : T cell CLL
T-LBL : T cell LBL
T-NHL : T cell NHL
TAE : Tris-acetate/EDTA electrophoresis buffer
TcR : T cell receptor
TcR-$\alpha\beta$: T cell receptor $\alpha\beta$ (classical TcR)
TcR-$\gamma\delta$: T cell receptor $\gamma\delta$ (alternative TcR)
TcR-CD3 : complex of TcR and CD3 chains
TdT : terminal deoxynucleotidyl transferase
TE : Tris-EDTA buffer
TEA : T early alpha
TES : Tris-EDTA-SDS buffer
TI : trypsin inhibitor
TLCK : tosyllysine chloromethyl ketone (protease inhibitor)
TNE : Tris-NaCl-EDTA buffer
TPCK : tosylphenylalanine chloromethyl ketone (protease inhibitor)
TRITC : tetramethylrhodamine isothiocyanate
TX : Texas red
v : volume
V : variable
VCR : vincristine
VLA antigen : very late activation antigen
VNR : vitronectin receptor
w : weight
WBC : white blood cells
weak Cyμ : weak cytoplasmic expression of μ Ig heavy chain
wk : week(s)
y : year(s)

DANKWOORD

Dit proefschrift is tot stand gekomen dankzij de medewerking van velen, zowel voor als achter de schermen. Ik wil hen allen dan ook op deze plaats hartelijk danken voor hun inzet en steun. Daarbij wil ik diverse personen met name in dit dankwoord betrekken.

Allereerst gaat mijn dank uit naar mijn promotor Professor Dr. R. Benner. Beste Rob, jij hebt de basis gecreëerd van waaruit dit onderzoek heeft kunnen plaatsvinden. Door het vertrouwen en de vrijheid die je me gaf, was het mogelijk een nieuwe researchlijn op te bouwen. Jouw houding heeft me gestimuleerd om de mogelijkheden zo goed mogelijk te benutten. Daarbij wist ik me altijd door jou gesteund. Niet alleen in voorspoedige tijden was je er, maar vooral ook in moeilijke tijden was je bereikbaar en bereid om mee te denken. Dit gevoel van steun en vriendschap hoop ik te blijven ervaren in onze samenwerking.

Professor Dr. A. Hagemeijer, beste Anne, dankzij de goede samenwerking met jou en de verschillende medewerkers van jouw groep is het mogelijk geweest het eerste gezamenlijke KWF-project uit te voeren, dat de aanzet heeft gegeven voor het humane immunologische onderzoek op onze afdeling. Ik ben dan ook zeer verheugd, dat je bereid was om mede mijn promotor te zijn.

Professor Dr. O. Vos dank ik voor zijn samenbindende en stimulerende rol binnen onze afdeling, als vaak stille, maar altijd krachtige steun op de achtergrond.

Professor Dr. J.J. van Rood en Professor Dr. B. Löwenberg wil ik danken dat zij zitting hebben willen nemen in de "kleine" promotiecommissie. Ik ben hen zeer erkentelijk voor hun bereidwilligheid om binnen een zeer kort tijdsbestek de manuscripten voor dit proefschrift te beoordelen.

Dankzij Ingrid Wolvers-Tettero en Marieke Comans-Bitter heb ik steeds vier extra rechterhanden gehad. Hun enorme inzet en enthousiasme, gecombineerd met gouden handen en ogen plus een grote bereidwilligheid om altijd en overal (in de trein, in de volkstuin of in het weekend) met het werk bezig te zijn, maakt dat ze niet alleen tijdens mijn promotie, maar ook in het leven van alledag mijn paranimfen zijn. Beste Ingrid en Marieke, jullie steun en hulp is onmisbaar! Verder is ook een woord van dank aan hun thuisfront op zijn plaats. Hans en Rob, bedankt voor jullie begrip en bereidheid om ook jullie steentje bij te dragen.

Mijn collega's Herbert Hooijkaas en Henk Adriaansen wil ik danken voor hun steun en collegialiteit. Beste Herbert, al in een vroeg stadium heb je meegeholpen het humane immunologisch onderzoek en de immunodiagnostiek op te bouwen. Met plezier denk ik terug aan de beginperiode, toen we samen "een hok" deelden. Het gezamenlijk werken aan de op- en uitbouw van het immunodiagnostisch laboratorium was een bijzondere ervaring. Ik ben dan ook blij dat je op de dag van mijn promotie als ceremonie-meester opnieuw de zaken regelt. In een wat later stadium kwam Henk ons team versterken. Beste Henk, ook jouw bereidwilligheid, inzet en steun waren onmisbaar voor de immunodiagnostiekgroep. In de loop der tijd heeft ieder zijn eigen taken en verantwoordelijkheden gekregen, maar de nauwe samenwerking en collegialiteit is binnen ons trio altijd blijven bestaan.

Dr. G.E. van Zanen en Dr. K. Hählen, beste George en Karel, de samenwerking tussen de

subafdeling Hematologie/Oncologie van de afdeling Kindergeneeskunde van het Sophia Kinderziekenhuis en onze afdeling heeft een belangrijke bijdrage geleverd aan de ontwikkeling van de immunodiagnostiek van leukemieën en maligne lymfomen. De prettige en vruchtbare samenwerking met jullie groep is in de loop der jaren steeds verder uitgegroeid en zal zeker gecontinueerd worden.

Dr. J. Borst, beste Jannie, ik heb je leren kennen en waarderen als een prettige en integere collega. Het is stimulerend om met je te "brainstormen" over de vele facetten van de immunologie. Ik hoop dan ook dat onze samenwerking van lange duur zal zijn.

Professor Dr. H.J. Neijens en Dr. R. de Groot, beste Herman en Ronald, sinds enkele jaren bestaat er een vruchtbare samenwerking op het gebied van immunodeficiënties bij kinderen. Immunopediatrie heeft mij altijd geboeid. Ik hoop dan ook dat ons gezamenlijk onderzoek meer inzicht zal geven in de achtergronden van primaire immunodeficiënties en dat de betreffende patiëntjes baat zullen hebben bij de resultaten.

Dr. G. Grosveld en Dr. A. de Klein, beste Gerard en Annelies, jullie wil ik bedanken voor het advies en de hulp die ik gekregen heb bij mijn eerste schreden op het "DNA-pad".

Prof. Dr. C. Terhorst en Dr. P. van den Elsen, beste Cox en Peter, jullie wil ik bedanken voor de mogelijkheid om in Boston onderzoekervaring op te doen. In alle opzichten was het een leerzame en boeiende periode.

Dr. E. van Wering en Dr. A. van der Does, beste Elisabeth en Anna, ik wil jullie en de Stichting Nederlandse Werkgroep Leukemie bij Kinderen (SNWLK) waarvan jullie deel uitmaken, danken voor de waardevolle samenwerking van de afgelopen jaren. Dit is een geweldige stimulans geweest voor het immunologische onderzoek bij kinderen met leukemie. Ook onze nieuwe gezamenlijke onderzoekplannen betreffende het opsporen van "minimal residual disease" lijken veelbelovend. Ik hoop dan ook dat onze gezamenlijke inspanningen mogen leiden tot een beter inzicht in de diagnostiek en behandeling van kinderen met leukemie.

Tar van Os en Cor van Dijk zijn op velerlei wijze betrokken geweest bij de visuele verslaglegging van de onderzoekresultaten en ideeën. Vaak was het veel en vaak was er haast, maar aan de kwaliteit van de figuren en dia's was dat nooit te merken. Hartelijk dank voor jullie zorg en inzet.

Tom Vermetten, Rein Smit, Piet Hartwijk, Mevrouw Godijn, Jopie Bolman, Elly Hoffman en Joke Bolman, zonder jullie steun achter de schermen is het goed functioneren van een afdeling als de onze onmogelijk. Daarom wil ik jullie op deze plaats bedanken voor jullie continue en betrouwbare ondersteuning.

Geertje de Korte, Jacqueline de Goey-van Dooren, Elly Schenkel en de laatste maanden vooral Rieke de Jager, jullie wil ik danken voor de uitstekende secretariële ondersteuning en vooral ook voor jullie inzet bij het verwerken van de teksten en de opmaak van de hoofdstukken van dit proefschrift.

In de afgelopen jaren hebben velen bijgedragen aan de opbouw en het optimaal functioneren van het immunodiagnostisch laboratorium. Dit laboratorium neemt een centrale plaats in binnen onze afdeling en is onontbeerlijk voor de medisch-immunologische research. Daarom dank ik René van den Beemd, Carla Borg, Annemiek Broos, Marieke Comans-Bitter, Ellen Cristen, Rian Deijkers-van Strien, Diana van den Goorbergh, Jacqueline de Groot-Pijnenburg, Sinka 't Hooft-Benne, Carin Kappetijn-van Tilborg, Manfred van Kerckhoven, Rianne van der Linde-Preesman, Jeroen te Marvelde, Hidde Osinga, Felix Rodriguez, Petra Soeting,

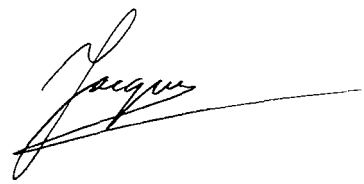
Annet Wierenga-Wolf, Anita Wijdenes-de Bresser, Annemarie Wijkhuijs en Ingrid Wolvers-Tettero voor hun inzet en enthousiasme ten behoeve van het immunodiagnostische werk.

Mijn directe medewerkers, Auke Beishuizen, Timo Breit, Marieke Comans-Bitter, Ellen Mol, Marie-Anne Verhoeven en Ingrid Wolvers-Tettero wil ik danken voor hun inzet en enthousiasme, waarmee ze hebben meegeholpen aan het afronden van dit proefschrift. Dat jullie bereid waren taken op je te nemen om mij te ontlasten, en bij te springen waar dat nodig was, maakte het voor mij mogelijk om dit proefschrift te voltooien. De sfeer waarin dit gebeurde, houdt een belofte in voor de toekomst.

Mijn dierbare ouders, "ons ma en ons pa", ben ik dankbaar voor hetgeen ik van ze heb meegekregen en geleerd. Mijn moeder was het voorbeeld in doorzettingsvermogen en inzet. In het tegemoet treden van moeilijke situaties was mijn vader het voorbeeld. Van hen beiden heb ik veel warme belangstelling voor mijn werk en steun in velerlei vorm mogen ondervinden. Vaak was ma nog enthousiaster over mijn onderzoekresultaten dan ikzelf en pa genoot in stilte mee. Aan een goed gesprek heeft het nooit ontbroken. Pa en ma, jullie positieve en reële levensvisie zal mij blijvend beïnvloeden.

En tenslotte, lieve Jeanette, niet alleen ik, maar ieder die jou van nabij kent, weet dat zonder jouw warmte, spontaniteit en wonderbaarlijke verdraagzaamheid dit proefschrift in deze vorm nooit tot stand was gekomen. De stelling "Belofte maakt schuld, ook voor een promovendus" is voor jou en voor ons samen bedoeld.

Nieuwerkerk aan den IJssel,
juni 1990



CURRICULUM VITAE

Jacobus Johannes Maria van Dongen (Jacques)

27 april 1955	: Geboren te Bergen op Zoom	
1967-1974	: Gymnasium β (Mollerlyceum, Bergen op Zoom)	
1974-1981	: Studie Geneeskunde (Erasmus Universiteit, Rotterdam)	
feb.-sept. 1977	: Keuze-practicum	: Afdeling Celbiologie en Genetica (o.l.v. Dr. R. Benner)
	Onderwerp	: Invloed van corticosteroiden op antilichaamvorming in het beenmerg
1977-1980	: Student-assistentschap	: Afdeling Pathologische Anatomie II (o.l.v. Prof. Dr. M.J. de Vries)
	Taak	: Onderwijs in de Algemene Ziekteleer voor 3e jaars studenten
feb. 1980	: Doctoraal examen Geneeskunde (Erasmus Universiteit Rotterdam)	
juni-juli 1981	: Research stage	: Afdeling Hepatologie, Katholieke Universiteit Leuven (o.l.v. Prof.Dr. K. Heirwegh, Prof.Dr. J. Fevery en Dr. N.B. Blankaert)
	Onderwerp	: Bilirubine metabolisme bij neonaten
juli 1981	: Artsexamen (Erasmus Universiteit Rotterdam)	
1981-heden	: Promotieonderzoek	: Afdeling Immunologie (o.l.v. Prof. Dr. R. Benner)
	Onderwerp	: Human T cell differentiation: basic aspects and their clinical applications
1984-heden	: Universitair Docent aan de Erasmus Universiteit en Consulent Immunologie voor het Academisch Ziekenhuis Rotterdam	
juni-aug. 1985	: Research stage	: Afdeling Moleculaire Immunologie (o.l.v. Prof. Dr. C. Terhorst en Dr. P. van den Elsen), Dana-Farber Cancer Institute, Harvard Medical School, Boston
	Onderwerp	: T cel receptor-CD3 complex gedurende de T cel differentiatie
okt.'86-jan.'87	: Assistentschap Kindergeneeskunde (Sophia Kinderziekenhuis, Rotterdam)	
nov. 1986	: Erkenning als Immunoloog door de Stichting voor Opleiding tot Medisch Biologisch Wetenschappelijk Onderzoeker	
nov. 1987	: Onderzoekprijs 1987 van de Erasmus Universiteit	
feb. 1988	: Klinisch Genootschapprijs 1987	
mei 1988	: Erkenning als Proefdierdeskundige (onderzoeker ex artikel 9 van de Wet op de dierproeven) door de Veterinaire Hoofdinspectie	
dec. 1988	: Verlening van "Aantekening Medische Immunologie" door het Convent Medische Immunologie van de Nederlandse Vereniging voor Immunologie	
april 1989	: Bekales Prize 1988 (Leucemia) van de Fondation Bekales (België)	

PUBLICATIONS (Original Articles, Book Chapters, and Reviews)

1. Benner R, Van Dongen JJM, Van Oudenaren A. Corticosteroids and the humoral immune response of mice:I. differential effect of corticosteroids upon antibody formation to sheep red blood cells in spleen and bone marrow. Cell Immunol 1978;41:52-61.

2. Van Dongen JJM, Hooijkaas H, Grosveld G, Hagemeijer A, Michiels JJ, Abels J, Van der Kwast ThH, Prins MEF, De Klein A. Case 31-1983: Richter's syndrome. N Engl J Med 1983;309:1582-3.

3. Van Dongen JJM, Hooijkaas H, Hählen K, Benne K, Bitter WM, Van der Linde-Preesman AA, Tettero ILM, Van de Rijn M, Hilgers J, Van Zanen GE, Hagemeijer A. Detection of minimal residual disease in TdT positive T cell malignancies by double immunofluorescence staining. In: Löwenberg B, Hagenbeek A, eds. Minimal residual disease in acute leukemia. Dordrecht: M Nijhoff Publishers, 1984:67-81.

4. Hooijkaas H, Van Dongen JJM, Hählen K, Van Zanen GE. Immunological characterization of cells in cerebrospinal fluid from patients with lymphoid malignancies. Lancet 1984;i:518-9.

5. Van Dongen JJM, Hooijkaas H, Hagemeijer A, Hählen K, Van Zanen GE, Lansdorp PM. Characterization and monitoring of the malignant cells in the blastic and chronic phase of chronic myeloid leukemia. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1984:711-4.

6. Van Dongen JJM, Hooijkaas H, Michiels JJ, Grosveld G, De Klein A, Van der Kwast ThH, Prins MEF, Abels J, Hagemeijer A. Richter's syndrome with different immunoglobulin light chains and different heavy chain gene rearrangements. Blood 1984;64:571-5.

7. De Groot CJ, Van Dongen JJM. Icterus van de pasgeborene: een koppig probleem. Ned Tijdschr Geneeskd 1984;128:1610-4.

8. Geurts van Kessel A, Tetteroo P, Van Agthoven T, Paulussen R, Van Dongen J, Hagemeijer A, Von dem Borne A. Localization of human myeloid-associated surface antigen detected by a panel of 20 monoclonal antibodies to the q12-qter region of chromosome 11. J Immunol 1984;133:1265-9.

9. Van Dongen JJM, Tettero ILM, Geurts van Kessel AHM, Versnel MA, Hooijkaas H, Hagemeijer A. Confirmation of the assignment of the gene coding for the BA-2 antigen to human chromosome 12. Hum Genet 1984;68:351-2.

10. Van Joost Th, Stolz E, Blog FB, Van der Kwast ThH, Vuzevski VD, Van Dongen JJM. B-cell markers in malignant B-cell lymphoma with scleroderma-like manifestation. J Cutan Pathol 1984;11:495-500.

11. Hooijkaas H, Van Dongen JJM, Hählen K, Van Zanen GE. Identification and monitoring of terminal deoxynucleotidyl transferase positive cells in the cerebrospinal fluid of children with lymphoid malignancies. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1985;32:875-8.

12. Van der Kwast ThH, Cristen EGM, Prins MEF, Van Dongen JJM, Hooijkaas H. Expression of B cell markers in centroblastic/centrocytic non-Hodgkin's lymphomas. In: Peeters H, ed. Protides of the biological fluids. Oxford: Pergamon Press, 1985;32:883-6.

13. Hoogsteden HC, Van Dongen JJM, Hooijkaas H, Dieges PH, Zondervan PE. Alveolitis in an asymptomatic pigeon breeder. Chest 1985;87:272.

14. Van Dongen JJM, Hooijkaas H, Hählen K, Van Zanen GE. The small subpopulation of T cell marker$^+$/TdT$^+$ cells in the human bone marrow may represent prothymocytes. Adv Exp Med Biol 1985;186:889-99.

15. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Benne K, Van Os TM, De Josselin de Jong J. Triple immunological staining using colloidal gold, fluorescein and rhodamine as labels. J Immunol Methods 1985;80:1-6.

16. Hoogsteden HC, Van Dongen JJM, Van der Kwast ThH, Hooijkaas H, Hilvering C. Bilateral exsudative pleuritis, an unusual pulmonary onset of mixed connective tissue disease. Respiration 1985;48:164-7.

17. Van Dongen JJM, Hooijkaas H, Comans-Bitter WM, Hählen K, De Klein A, Van Zanen GE, Van 't Veer MB, Abels J, Benner R. Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR, and a T cell marker may represent prothymocytes. J Immunol 1985;135:3144-50.

18. Van Dongen JJM, Versnel MA, Geurts van Kessel AHM, Smit EME, Hooijkaas H, Hagemeijer A. Immunoglobulin chain gene expression in Burkitt lymphoma derived cell lines. In: Gale RP, Golde DW, eds. UCLA symposia on molecular and cellular biology, New Series, Vol.28: Leukemia: recent advances in biology and treatment. New York: Alan R. Liss, 1985:481-9.

19. Versnel MA, Van Dongen JJM, Geurts van Kessel AHM, De Klein A, Bos NA, Hagemeijer A. Expression of the human immunoglobulin heavy chain gene of the 14q+ chromosome in a t(8;14) positive Burkitt lymphoma cell line demonstrated in somatic cell hybrids. Cancer Genet Cytogenet 1986;19:321-30.

20. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Detection of minimal residual acute lymphoblastic leukemia by immunological marker analysis: possibilities and limitations. In: Hagenbeek A, Löwenberg B, eds. Minimal residual disease in acute leukemia 1986. Dordrecht: M Nijhoff Publishers, 1986:113-33.

21. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen H, Van Zanen GE, De Pauw BE, Van der Valk P, Meijer CJLM. Immunologisch karakteriseren van leukemieën en non-Hodgkin lymfomen. Tijdschr Kindergeneeskd 1986;54:29-41.

22. Van Dongen JJM, Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE. Opsporen van lage aantallen maligne cellen bij patiënten met een leukemie of non-Hodgkin lymfoom: gebruik van immunologische markers. Tijdschr Kindergeneeskd 1986;54:41-5.

23. Hooijkaas H, Adriaansen HJ, Hählen K, Van Zanen GE, Van Dongen JJM. Terminaal deoxynucleotidyl transferase (TdT) positieve cellen in de liquor cerebrospinalis van kinderen met een leukemie of non-Hodgkin lymfoom: implicaties voor de diagnose centraal zenuwstelsel leukemie. Tijdschr Kindergeneeskd 1986;54:46-50.

24. Van Dongen JJM, Geurts van Kessel AHM, Wolvers-Tettero ILM, Versnel MA, Van Oudenaren A, Schoenmaker E, Hagemeijer A. Construction of human-mouse T cell hybrids that express human T cell associated surface antigens and allow the chromosomal localization of these antigens. J Immunol 1986;137:1047-53.

25. Hagemeijer A, Bolhuis RLH, Van Dongen JJM, Sizoo W. Simultaneous occurrence of acute monoblastic leukemia and an abnormal B-cell clone with both cell types characterized by specific cytogenetic and immunological markers. Scand J Haematol 1986;36:484-91.

26. Zijlstra FJ, Vincent JE, Van den Berg B, Hoogsteden HC, Neijens HJ, Van Dongen JJM. Pulmonary alveolar proteinosis: determination of prostaglandins and leukotrienes in lavage fluid. Lung 1987;165:79-89.

27. Hagemeijer A, Van Dongen JJM, Slater RM, Van 't Veer MB, Behrendt H, Hählen K, Sizoo W, Abels J. Characterization of the blast cells in acute leukemia with translocation (4;11): report of eight additional cases and of one case with a variant translocation. Leukemia 1987;1:24-31.

28. Van Dongen JJM, Quertermous T, Bartram CR, Gold DP, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, De Klein A, Raghavachar A, Ganser A, Duby AD, Seidman JG, Van den Elsen P, Terhorst C. The T cell receptor - CD3 complex during early T cell differentiation: analysis of immature T cell acute lymphoblastic leukemias (T-ALL) at DNA, RNA and cell membrane level. J Immunol 1987;138:1260-9.

29. Gold DP, Van Dongen JJM, Morton CC, Bruns GAP, Van den Elsen P, Geurts van Kessel AHM, Terhorst C. The gene encoding the ϵ subunit of the T3/T-cell receptor complex maps to chromosome 11 in humans and to chromosome 9 in mice. Proc Natl Acad Sci USA 1987;84:1664-8.

30. Quertermous T, Strauss WM, Van Dongen JJM, Seidman JG. Human T-cell gamma chain joining regions and T cell development. J Immunol 1987;138:2687-90.

31. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunological marker analysis of cells in the various hematopoietic differentiation stages and their malignant counterparts. In: Ruiter DJ, Fleuren GJ, Warnaar SO, eds. Application of monoclonal antibodies in tumor pathology. Dordrecht: M Nijhoff Publishers, 1987:87-116.

32. Hooijkaas H, Adriaansen HJ, Hählen K, Dekker I, Van Zanen GE, Van Dongen JJM. The presence or absence of terminal deoxynucleotidyl transferase (TdT) positive cells in the cerebrospinal fluid (CSF) has implications for the diagnosis of meningeal involvement in patients with acute lymphoblastic leukemia. In: Pruzanski W, Seligmann M, eds. Clinical immunology. Amsterdam: Elsevier Science, 1987:431-4.

33. Van Dongen JJM, Quertermous T, Bartram CR, Wolvers-Tettero ILM, Comans-Bitter WM, Hooijkaas H, Adriaansen HJ, Terhorst C. The T-cell receptor-CD3 complex during early T-cell differentiation. In: McMichael AJ, Beverly PCL, Cobbold S, Crumpton ML, Gilks W, Gotch FM, Hogg N, Horton M, Ling N, MacLennan ICM, Mason DY, Milstein C, Spiegelhalter D, Waldmann H, eds. Leucocyte typing III: white cell differentiation antigens. Oxford: Oxford University Press, 1987:198-9.

34. Tetteroo PAT, Visser FJ, Van Dongen JJM, Geurts van Kessel AHM. Expression of the common beta chain of LFA-1, CR3, p150.95 (CD18) in human-mouse somatic cell hybrids. In: McMichael AJ, Beverly PCL, Cobbold S, Crumpton ML, Gilks W, Gotch FM, Hogg N, Horton M, Ling N, MacLennan ICM, Mason DY, Milstein C, Spiegelhalter D, Waldmann H, eds. Leucocyte typing III: white cell differentiation antigens. Oxford: Oxford University Press, 1987:860-3.

35. Von dem Borne AEGKr, Van Dongen JJM, Hagemeijer A, Halie MR, Den Ottolander GJ. Celkenmerken en laboratoriumonderzoek. In: Halie MR, Von dem Borne AEGKr, eds. Nederlands leerboek der hematologie. Utrecht: Wetenschappelijke Uitgever Bunge, 1987:8-43.

36. Van Dongen JJM. Analysis of immunoglobulin genes and T cell receptor genes as a diagnostic tool for the detection of lymphoid malignancies. Neth J Med 1987;31:201-9.

37. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. De betekenis van immunologische marker analyse voor hemato-oncologische ziekten bij kinderen. IKR Bulletin 1987;11(4):12-7.

38. Van Dongen JJM, Wolvers-Tettero ILM, Seidman JG, Ang S-L, De Vries EFR, Borst J. Two types of gamma T cell receptors expressed by T cell acute lymphoblastic leukemias. Eur J Immunol 1987;17:1719-28.

39. Van Dongen JJM, Comans-Bitter WM. Phenotypic and genotypic characteristics of the human prothymocyte. Immunol Res 1987;6:250-62.

40. Collard JG, Van de Poll M, Scheffer A, Roos E, Hopman AHM, Geurts van Kessel AHM, Van Dongen JJM. Genes involved in invasion and metastasis are located on human chromosome 7. Cancer Res 1987;47:6666-70.

41. Van Dongen JJM, Benner R. Nobelprijs voor de Geneeskunde 1987. Ned Tijdschr Geneeskd 1987;52:2399-400.

42. Hooijkaas H, Adriaansen HJ, Van Dongen JJM. Detection of central nervous system (CNS) involvement in patients with a leukemia or non-Hodgkin lymphoma by immunological marker analysis of cerebrospinal fluid (CSF) cells. In: Bennett JM, Foon KA, eds. Immunologic approaches to the classification and management of lymphomas and leukemias. Dordrecht: M Nijhoff Publishers, 1988:149-71.

43. Ryan DH, Van Dongen JJM. Detection of residual disease in acute leukemia using monoclonal antibodies. In: Bennett JM, Foon KA, eds. Immunologic approaches to the classification and management of lymphomas and leukemias. Dordrecht: M Nijhoff Publishers, 1988:173-207.

44. Adriaansen HJ, Van Dongen JJM, Hooijkaas H, Hählen K, Van 't Veer MB, Löwenberg B, Hagemeijer A. Translocation (6;9) may be associated with a specific TdT positive immunological phenotype in ANLL. Leukemia 1988;2:136-40.

45. Van Dongen JJM, Krissansen GW, Wolvers-Tettero ILM, Comans-Bitter WM, Adriaansen HJ, Hooijkaas H, Van Wering ER, Terhorst C. Cytoplasmic expression of the CD3 antigen as diagnostic marker for immature T cell malignancies. Blood 1988;71:603-12.

46. Michiels JJ, Adriaansen HJ, Hagemeijer A, Hooijkaas H, Van Dongen JJM, Abels J. TdT positive B cell acute lymphoblastic leukemia with Burkitt characteristics. Br J Haematol 1988;68:423-6.

47. Hoogsteden HC, Van Dongen JJM, Bienfait MF, Hilvering C. Bronchoalveolaire lavage bij patiënten met extrapulmonale sarcoidose. Bulletin Fijnvlokkige Longafwijkingen 1988:12-5.

48. Van Dongen JJM. Immunoglobuline-genen en T-cel-receptor-genen en de expressie van immunologische markers: I. de opbouw van het specifieke immuunsysteem. Ned Tijschr Geneeskd 1988;132:855-62.

49. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunoglobuline-genen en T-cel-receptor-genen en de expressie van immunologische markers: II. immunologische diagnostiek van maligne lymfatische ziekten. Ned Tijdschr Geneeskd 1988;132:862-8.

50. Borst J, Van Dongen JJM, Bolhuis RLH, Peters PJ, Hafler DA, De Vries E, Van de Griend RJ. Distinct molecular forms of human T cell receptor gamma/delta detected on viable T cells by a monoclonal antibody. J Exp Med 1988;167:1625-44.

51. Hoogsteden HC, Van Dongen JJM, Adriaansen HJ, Hooijkaas H, Delahaye M, Hop W, Hilvering C. Bronchoalveolar lavage in patients presenting with extrapulmonary sarcoidosis. Chest 1988;94:115-8.

52. Frenkel J, Neijens HJ, Den Hollander JC, Wolvers-Tettero ILM, Van Dongen JJM. Oligoclonal T cell proliferative disorder in combined immunodeficiency. Pediatr Res 1988;24:622-7.

53. Hoogsteden HC, Van Hal PTW, Van Dongen JJM. Immunologische markers van cellen in bronchoalveolaire lavage (BAL) en perifeer bloed (PB) bij interstitiële longziekten. Bulletin Fijnvlekkige Longafwijkingen 1988:57-60.

54. Comans-Bitter WM, Van Lom K, Van Dongen JJM. Immunologische marker-analyse van hematopoiëtische cellen: immunofluorescentie en immunogoud-zilverversterking labelingen. Analyse 1988;43:283-92.

55. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunophenotyping of leukemias and non-Hodgkin lymphomas: immunological markers and their CD codes. Neth J Med 1988;33:298-314.

56. Van de Merwe JP, Frenkel J, Van Dongen JJM. Te vaak infecties: gestoorde weerstand? In: Aulbers BJM, Buntinx F, Neomagus GJH, Verdenias W, Van der Voort JPM, Van Weel C, eds. Jaarboek huisartsgeneeskunde 1989-1990. Utrecht: Wetenschappelijke Uitgeverij Bunge, 1989:79-100.

57. Tersmette M, Van Dongen JJM, Clapham PR, De Goede REY, Wolvers-Tettero ILM, Geurts van Kessel AHM, Huisman JG, Weiss RA, Miedema F. Human immunodeficiency virus infection studied in CD4-expressing human murine T cell hybrids. Virology 1989;168:267-73.

58. Hoogsteden HC, Van Dongen JJM, Van Hal PThW, Delahaye M, Hop W, Hilvering C. Phenotype of blood monocytes and alveolar macrophages in interstitial lung diseases. Chest 1989;95:574-7.

59. Schultz FW, Van Dongen JJM, Hählen K, Hagenbeek A. Time-history of the malignant population in the peripheral blood of a child with T cell acute lymphoblastic leukemia: a pilot study. Comput Math Applic 1989;18:929-36.

60. Frenkel J, Van Dongen JJM, Benner R. Immunogenetica. In: Pronk JC, Geraedts JPM, Anders GJPA, Eriksson AW, Niermeijer MF, eds. Medische genetica. Utrecht: Wetenschappelijke Uitgeverij Bunge, 1989:259-93.

61. Van Dongen JJM, Wolvers-Tettero ILM, Wassenaar F, Borst J, Van den Elsen P. Rearrangement and expression of T cell receptor delta genes in T cell acute lymphoblastic leukemias. Blood 1989;74:334-42.

62. Hooijkaas H, Hählen K, Adriaansen HJ, Dekker I, Van Zanen GE, Van Dongen JJM. Terminal deoxynucleotidyl transferase (TdT) positive cells in cerebrospinal fluid and the development of overt central nervous system leukemia: a five year follow-up study in 113 children with a TdT positive leukemia or non-Hodgkin's lymphoma. Blood 1989;74:416-22.

63. Borst J, Wicherink A, Van Dongen JJM, De Vries E, Comans-Bitter WM, Wassenaar F, Van den Elsen P. Non-random expression of T cell receptor γ and δ variable gene segments in functional T lymphocyte clones from human peripheral blood. Eur J Immunol 1989;19:1559-68.

64. Michiels JJ, Van Dongen JJM, Hagemeijer A, Sonneveld P, Ploemacher RE, Adriaansen HJ, Van der Kwast ThH, Brederoo P, Abels J. Richter's syndrome with identical immunoglobulin gene rearrangements in the chronic lymphocytic leukemia and the supervening non-Hodgkin lymphoma. Leukemia 1989;3:819-24.

65. Neijens HJ, Frenkel J, De Muinck Keizer-Schrama SMPF, Dzoljic-Danilovic G, Meradje M, Van Dongen JJM. Invasive Aspergillus infections in chronic granulomatous disease: successful treatment of two patients with itraconazole. J Pediatr 1989;115:1016-9.

66. Adriaansen HJ, Hooijkaas H, Kappers-Klunne MC, Hählen K, Van 't Veer MB, Van Dongen JJM. Double marker analysis for terminal deoxynucleotidyl transferase and myeloid antigens in acute nonlymphocytic leukemia patients and healthy subjects. Haematol Blood Transfus 1990;33:41-9.

67. Adriaansen HJ, Wijdenes-de Bresser JHFM, Van Drunen-Schoenmaker E, Geurts van Kessel AHM, Van Dongen JJM. Expression of the myeloid differentiation antigen CD33 depends on the presence of human chromosome 19 in human-mouse hybrids. Ann Hum Genet 1990;54:115-9.

68. Smit MJM, De Groot R, Van Dongen JJM, Van der Voort E, Neijens HJ, Whitfield LR. Trimetrexate efficacy and pharmacokinetics during treatment of refractory *Pneumocystis carinii* pneumonia in an infant with severe combined immunodeficiency disease. Pediatr Inject Dis J 1990;9:212-14.

69. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunologische markers en hun CD codes. In: Adriaansen HJ, Van Dongen JJM, eds. Klinische toepassingen van flowcytometrie. Rotterdam: Department of Immunology, Erasmus University Rotterdam, 1990:19-40.

70. Adriaansen HJ, Hooijkaas H, Van Dongen JJM. Immunologische markeranalyse voor de diagnostiek van leukemieën en non-Hodgkin lymfomen. In: Adriaansen HJ, Van Dongen JJM, eds. Klinische toepassingen van flowcytometrie. Rotterdam: Department of Immunology, Erasmus University Rotterdam, 1990:41-57.

71. Van Dongen JJM, Adriaansen HJ, Hooijkaas H. Immunologische marker analyse voor de diagnostiek van primaire immunodeficiënties en deficiënties van differentiatie antigenen. In: Adriaansen HJ, Van Dongen JJM, eds. Klinische toepassingen van flowcytometrie. Rotterdam: Department of Immunology, Erasmus University Rotterdam, 1990:99-114.

72. Horst E, Meijer CJLM, Radaszkiewicz T, Van Dongen JJM, Pieters R, Figdor CG, Hooftman A, Pals ST. Expression of a human homing receptor (CD44) in lymphoid malignancies and related stages of lymphoid development. Leukemia 1990, in press.

73. Ludwig W-D, Van Dongen JJM, Thiel E, Teichmann JV, Hofmann J, Riehm H. Possibilities and limitations of immunological marker analyses for the detection of minimal residual disease in childhood acute lymphoblastic leukemia. Onkologie 1990, in press.

74. Mason DY, Comans-Bitter WM, Cordell JL, Verhoeven M-AJ, Van Dongen JJM. Antibody L26 recognizes an intracellular epitope on the B cell associated CD20 antigen. Am J Pathol 1990, in press.

75. Adriaansen HJ, Van Dongen JJM, Kappers-Klunne C, Hählen K, Van 't Veer MB, Wijdenes-de Bresser JHFM, Holdrinet ACJM, Harthoorn-Lasthuizen EJ, Abels J, Hooijkaas H. Terminal deoxynucleotidyl transferase positive subpopulations occur in the majority of ANLL: implications for the detection of minimal residual disease. Leukemia 1990, in press.

76. Borst J, Van Dongen JJM, De Vries E, Comans-Bitter WM, Van Tol MJD, Vossen JM, Kurrle R. BMA031, a monoclonal antibody specifically suited to identify the T cell receptor $\alpha\beta$/CD3 complex on viable human T lymphocytes in normal and disease states. Hum Immunol 1990, in press.

77. Van Dongen JJM, Comans-Bitter WM, Wolvers-Tettero ILM, Borst J. Development of human T lymphocytes and their thymus-dependency. Thymus, in press.

78. Van Dongen JJM, Wolvers-Tettero ILM. Analysis of immunoglobulin and T cell receptor genes: possibilities and limitations in the diagnosis and management of lymphoproliferative diseases and related disorders. Clin Chim Acta, accepted for publication.

79. Campana D, Van Dongen JJM, Mehta A, Couston-Smith E, Wolvers-Tettero ILM, Ganeshaguru K, Janossy G. The stages of T cell receptor protein expression in T-cell acute lymphoblastic leukemia. Submitted for publication.

80. Van Dongen JJM, Comans-Bitter WM, Friedrich W, Neijens HJ, Belohradsky BH, Kohn Th, Hagemeijer A, Borst J. Analysis of patients with DiGeorge anomaly provides evidence for extrathymic development of TcR-$\gamma\delta^+$ lymphocytes in man. Submitted for publication.