HETEROGENEITY IN ACUTE MYELOID LEUKEMIA: BASIC AND DIAGNOSTIC STUDIES

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HETEROGENEITY IN ACUTE MYELOID LEUKEMIA

Basic and diagnostic studies

HETEROGENITEIT VAN ACUTE MYELOIDE LEUKEMIE

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Aan Petra
Aan mijn ouders

HETEROGENEITY IN ACUTE MYELOID LEUKEMIA

Basic and diagnostic studies

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CHAPTER 1

GENERAL INTRODUCTION

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GENERAL INTRODUCTION

1.1 NORMAL AND ABNORMAL HEMATOPOIESIS

In smears of human peripheral blood (PB) at least seven different cell types can be recognized, i.e. erythrocytes, platelets, neutrophils, basophils, eosinophils, monocytes, and lymphocytes. Each cell type has its own specific function and is involved in e.g. gas transport, hemostasis, immunity, and inflammation (1). The formation of different blood cells (hematopoiesis) primarily occurs in the bone marrow (BM). The committed precursor cells and their progeny are derived from a small population of immature pluripotent hematopoietic stem cells (2-7). These pluripotent stem cells have the ability to remain quiescent and to undergo self-renewal (6-8). Hematopoiesis is tightly regulated and a balance between cell formation and cell destruction is maintained (4,6-9). In case of increased demands, such as in bleeding or infection, a compensatory increase in cell production will occur.

During the last two decades major progress has been made in the understanding of the molecular processes which are involved in hematopoiesis (4,9,10). Blood cell formation is regulated by cell-to-cell interactions in the BM and by several soluble regulatory glycoprotein molecules, the hematopoietic growth factors (4,9-14). These hematopoietic growth factors as well as several other cytokines are involved in the maintenance of cell viability, induction of proliferation, differentiation, and maturation of the hematopoietic cells. Furthermore, they can enhance several functional activities of mature PB cells, especially in host responses to infection or antigenic challenge (11,15,16). Some properties of cytokines involved in hematopoiesis are summarized in Table 1 (14,17-99). Comparable with other systems in biology, like the complement system and the blood clotting system, there is a complex network of interactions between cytokines and various cell types, i.e. stromal cells, endothelial cells, and hematopoietic cells (4,9-11,14-16,100-102). The cascade of interactions has been extensively reviewed by others and is beyond the scope of this thesis (4,9-11,14-16,102). Figure 1 summarizes the target cells of hematopoietic growth factors and other cytokines during hematopoiesis (4,11,14-17,51,71,75,81,86,89-91,102-123). It should be emphasized that this figure is a hypothetical and simplified summary of complex interactions in which synergism, antagonism, suppression, and autoregulation play a role. An example of synergism comes from experiments with the recently isolated and cloned stem cell factor (SCF), also known as mast cell growth factor or c-*kit* ligand (105-109,113,114,119). These experiments indicate that SCF synergistically interacts with various cytokines to augment the proliferative capacity of hematopoietic progenitor cells, precursor B cells, and mast cells (Figure 1).

TABLE 1. Cytokines involved in hematopoiesis.

Factor	Molecular Mass (kDa)[a]	Gene localization	Cellular source	References
Hematopoietic growth factors				
SCF	18-35[b]	12q22-q24	fibroblasts	17-21
IL-3[c]	15-25	5q23-q31	T cells	14,22-25
GM-CSF	18-24	5q23-q31	T cells, monocytes, macrophages, fibroblasts, endothelial cells	14,25-30
G-CSF	18-22	17q11.2-q21	monocytes, macrophages, fibroblasts, endothelial cells	14,28-35
M-CSF	35-45[b]	1p13-p21	monocytes, macrophages, fibroblasts, endothelial cells	14,30,35-40
Erythropoietin	34-39	7q21.3-q22.1	peritubular cells of the kidney	14,41-46
Other cytokines				
IL-1α/β[d]	17	2q12-q21	lymphocytes, monocytes, macrophages, fibroblasts, endothelial cells	47-51
IL-2	15	4q26-q27	T cells	52-56
IL-4	15	5q23-q31	T cells, mast cells	57-61
IL-5	20[b]	5q23.3-q31	T cells	61-65
IL-6	21-26	7p15-p21	T cells, monocytes, macrophages, fibroblasts, endothelial cells	66-71
IL-7	17-25	8q12-q13	fibroblasts	72-75
IL-8	8-11	4q13-q21	T cells, monocytes,macrophages, fibroblatst, endothelial cells	76-79
IL-9	37-40	5q31-q32	T cells	80-83
IL-10	17-21	1	T cells, monocytes, macrophages B cells	84-86
IL-11	23	19q13.3-q13.4	fibroblasts	87-91
TNFα	17	6p21.3	T cells, monocytes, macrophages	92-94
TNFβ	18-25	6p21.3	T cells, monocytes, macrophages	93-95
TGFβ1	12[b]	19p13.1	lymphocytes, monocytes, macrophages, platelets, fibroblasts	96-98

a. Due to variable glycosylation and sialylation of the cytokines molecular mass is often heterogeneously.
b. SCF, M-CSF, IL-5, and TGFβ have also been isolated as homodimers. The molecular mass of the monomeric forms is given.
c. Interleukin classification for IL-1 to IL-10 according to the international union of immunological societies nomenclature subcommittee on interleukin designation (99).
d. Two distinct IL-1 molecules, IL-1α and IL-1β, have been isolated.
Abbreviations used: kDa = kilo Dalton, SCF = stem cell factor, IL = interleukin, GM-CSF = granulocyte macrophage colony stimulating factor, G-CSF = granulocyte CSF, M-CSF = macrophage CSF, TNF = tumor necrosis factor, TGF = transforming growth factor.

The down regulatory actions of some cytokines on hematopoiesis are not indicated in Figure 1. Growth inhibitory influences have been demonstrated for interleukin (IL)-4, IL-10, tumor necrosis factor (TNF), transforming growth factor (TGF)β as well as interferon-γ (15,60,86,124-131). As indicated in Table 1, T lymphocytes, monocytes, macrophages, endothelial cells, and fibroblasts represent the major sources of the cytokines. Cytokines produced by monocytes and macrophages, such as IL-1 and TNF, can cause the release of cytokines from other cell types, which illustrates the central role of the monocyte-macrophage lineage in cytokine release (15,16,51,124).

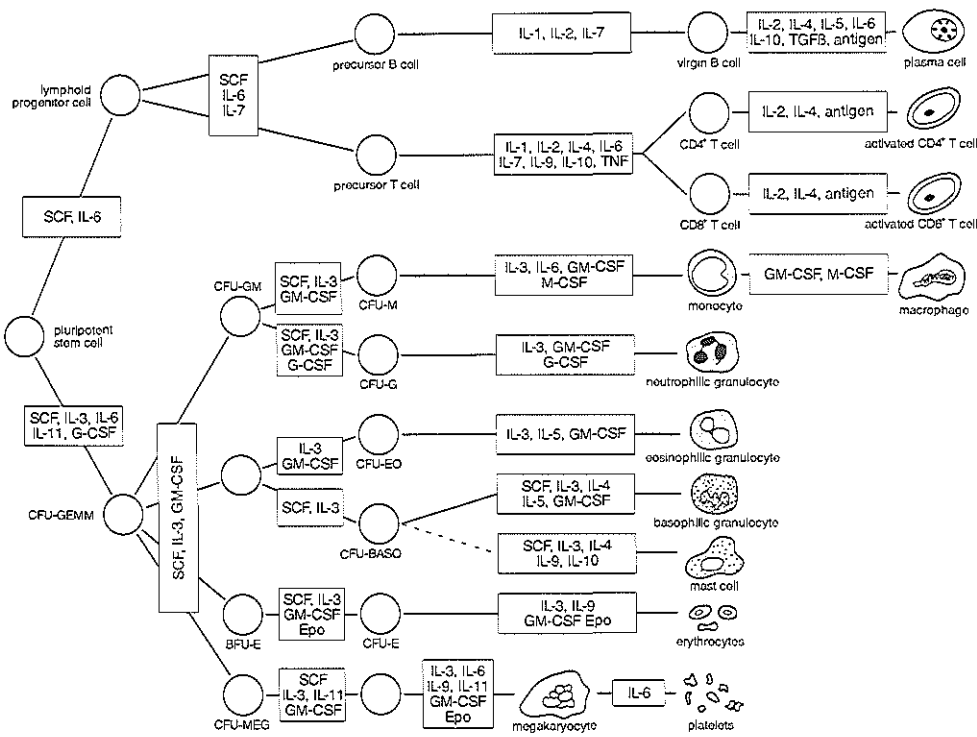

Figure 1. Hypothetical scheme of human hematopoiesis. Hematopoietic growth factors and some other cytokines which can induce differentiation, maturation, or proliferation of certain cell types during hematopoiesis are indicated (white boxes). Data on IL-7 and SCF interactions on precursor lymphoid cells as well as the actions of IL-10 during mast cell differentiation were partly obtained in mice (72,73,75,113,114,120). In mice IL-11 proved to have lymphopoietic properties (87), but at present, reports on the action of IL-11 during human hematopoiesis are restricted to progenitor cells and megakaryocytopoiesis (89-91). Although mast cells were shown to originate from BM derived pluripotent progenitor cells their relation with the other cell lineages has not been proved (119,120). BFU-E = erythrocyte burst forming unit, CFU = colony forming unit, CFU-Baso = basophil CFU, CFU-E = erythrocyte CFU, CFU-EO = eosinophil CFU, CFU-G = granulocyte CFU, CFU-GEMM = granulocyte erythrocyte macrophage megakaryocyte CFU, CFU-GM = granulocyte macrophage CFU, CFU-M = macrophage CFU, Epo = erythropoietin. For the other abbreviations used see legend of Table 1. Adapted from references 4,11,14-17,51,71,75,81,86,89-91,102-123.

For each cytokine an unique membrane receptor exists, which can be found on responding cells. Cloning of the receptors for the cytokines listed in Table 1 revealed a significant homology for several receptors (51,56,96,132-146). These cytokine receptors have been designated as the hematopoietin receptor superfamily (147-149). Complex intracellular mechanisms are involved in signalling through the receptors (150-152). Probably, there are some common control pathways within the cell, which may explain synergistic as well as antagonistic effects of various cytokines (150).

Disturbances of hematopoiesis can result in quantitative and/or qualitative alterations in BM and PB. These alterations may be manifested in one cell lineage, e.g. anemia in

thalassemia, or in several different cell series, e.g. pancytopenia due a toxic agent. The etiology of the various disorders is manyfold, including deficiency states, toxic effects, autoreactive processes, infectious diseases, hereditary disorders, and genetic alterations (1). The latter can result in disturbances of growth and differentiation, which are manifested as aplasia, dysplasia, or malignancy (10,153-155).

Depending on the organ system primarily involved, i.e. BM, PB, or lymphoid tissues, the malignancies of the hematopoietic system are denominated leukemias or lymphomas, respectively. According to clinical presentation the leukemias are divided in acute leukemias which, if untreated, usually cause death in weeks or months and chronic leukemias which, if untreated, cause death in months or years (1). In general, the acute leukemias are characterized by excessive proliferation and abnormal differentiation of immature cell types in BM and PB leading to disturbed hematopoiesis (1,156). They comprise a heterogeneous group of conditions which differ in biology and prognosis. Depending on the cell lineage(s) which are involved in the leukemic process the acute leukemias can be divided into acute lymphoblastic leukemias (ALL) and acute non-lymphocytic leukemias (ANLL) (1,156-159). The latter include the acute myeloid leukemias (AML) and acute undifferentiated leukemias (AUL). According to the recently redefined morphological classification of acute leukemias, the far majority (>99%) represent either AML or ALL and only a small fraction (<1%) represent AUL (159,160). Within each group of acute leukemias considerable heterogeneity is found. This heterogeneity is manifested in a patient-to-patient variation in clinical presentation, cytomorphology, cytogenetics, response to therapy, and prognosis (156-161). Furthermore, in most AML patients the leukemic cells partly retain the ability to differentiate and mature which results in a marked phenotypic heterogeneity within each leukemia. The recognition of subgroups of biologically similar cases as well as the identification of different cell subpopulations within each acute leukemia is important for a better understanding of the leukemic processes. Such knowledge may ultimately lead to different therapeutic approaches in well-defined subgroups and can be used to define which leukemic cell subpopulations are important for leukemia growth and regrowth during relapse.

1.2 EPIDEMIOLOGY OF AML

AML occur at all ages with an overall incidence of 1 to 3 per 100,000 persons per year (162-164). The incidence rates are greater for males than females and for whites than non-whites (162-164). Furthermore, international comparisons showed geographic differences in incidence rates (164,165). In the Netherlands AML has been reported to occur in 2.4 per 100,000 inhabitants each year (166). The results of a 15-years study of the Dutch childhood leukemia study group (DCLSG) and a 10-years study of the Eindhoven Cancer Registry Office are outlined in Figure 2 (166,167). Similar to other studies there is an age specific incidence of AML (162,164,166,168). The small peak in the incidence of AML during infancy is caused by congenital leukemias which relatively frequently represent AML (164,167-169).

However, during childhood the most common leukemia is ALL which has a sharp peak of disease incidence at age 3-5 (Figure 2) (162,164,167,168). The incidence of AML during childhood and young adulthood is less than 1 per 100,000 each year. Above the age of 30 the incidence of AML rises from 1 per 100,000 per year to more than 10 AML cases per 100,000 persons per year above the age of 70 (Figure 2). It is clear that in adults AML is the most frequent form of acute leukemia. If all types of leukemia are taken into account, AML is the most common type of leukemia in the middle-aged group (164,166). During the last decades the incidence of AML has been reported to increase (162,170). This increase as well as sex, age, and geographic differences in the incidence rates of AML support the hypothesis that occupational and environmental exposures are potentially important in the etiology. In general, AML which arises *de novo* is denominated primary AML, whereas secondary AML arises from preleukemic disorders and/or after leukemogenic exposures (155,156,160).

Risk factors for the development of AML which have been consistently reported in the literature are summarized in Table 2. It should be noticed that many studies are limited by inadequate sample size, imprecise case definition, or inadequate exposure measure (164, 171). Children with Down's syndrome or Klinefelter's syndrome are at increased risk for childhood leukemia, especially congenital leukemia (163,164,168,172-174). In other genetic syndromes abnormalities of DNA repair processes predispose for the development of AML

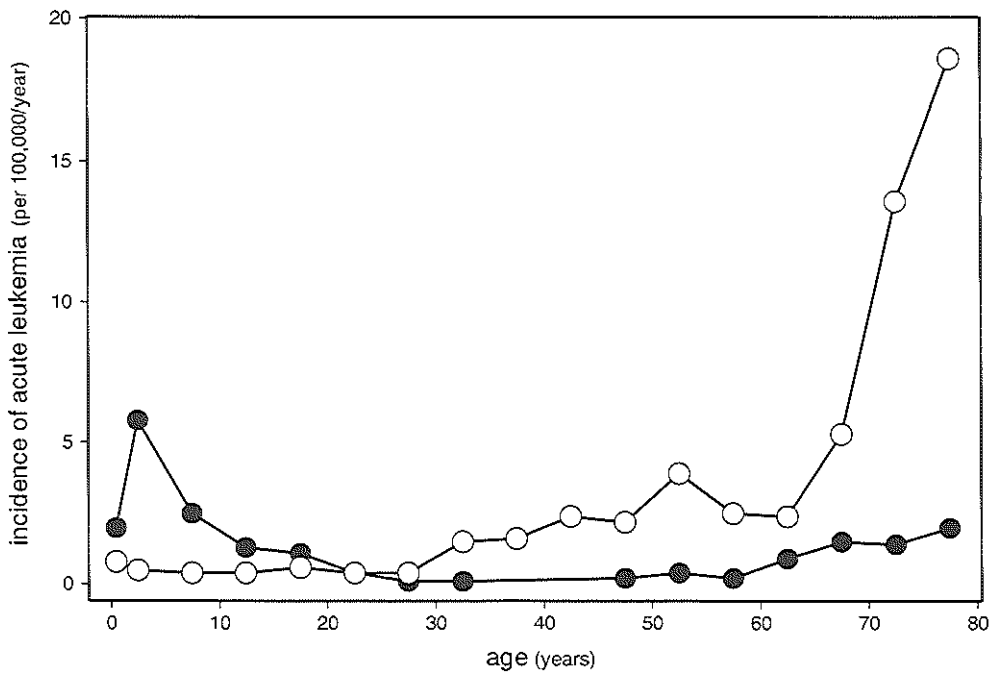

Figure 2. Age specific incidence of AML (open symbols) and ALL (closed symbols) in the Netherlands according to DCLSG (0-15 years) and Eindhoven cancer registry (> 15 years) (166,167). Incidence in first year of life and in the 1-4 years group is given. Subsequent values represent the mean of a 5 years age group.

TABLE 2. Potential risk factors for AML, as reported in the literature[a].

Genetic factors	- Down's syndrome - Klinefelter's syndrome - ataxia-teleangiectasia - Bloom's syndrome - Fanconi's anemia	Chemicals	- benzene - other petroleum products and solvents - pesticides
Radiation	- atomic explosions or fall-out from nuclear testing - therapeutic irradiation - X-ray contrast agents, especially thorotrast - background radiation, especially radon inhalation	Cigarette smoking	
		Drugs	- alkylating agents - nitrosoureas - procarbazine

a. References 163,164,168,172-202.

(163,164,168,172,174,175). Studies on Japanese survivors of the atomic bomb as well as on military personnel or civilians exposed during nuclear testing demonstrated an increased risk incidence of AML for those exposed to ionizing radiation (163,164,176-178). However, an association between increased risk of childhood leukemia and residence in the southern countries of Utah during aboveground nuclear testing at the Nevada test site (178), could not be confirmed on closer examination of available data (179). In two large studies on people who have been employed at or lived nearby nuclear plants, there was no evidence of an excess of leukemia (180,181). Follow-up of patients irradiated for various diseases, such as ankylosing spondylitis or cervical cancer revealed that AML risk increased with the exposure doses but that the incidence curve flattened at higher doses (163,164,182-184). The latter has been attributed to a cell killing effect at these high doses (184). Whether natural background radiation at home from radon is a causative factor in the induction of AML needs further investigation (185,186). Benzene is probably the best known and most widely occurring leukemogen and, together with other potential leukemogens, it might account for increased AML in cigarette smokers (163,164,187-193). Increased AML risk has also been suggested for organic solvent or pesticide exposures (194,195). There have been many reports of secondary AML in patients treated for other malignancies with combined chemotherapy and/or irradiation (196-202). Especially alkylating drugs have a high leukemogenic potential and these agents probably cause the relatively high incidence of secondary AML in patients treated for Hodgkin's disease (196,197,200). Chromosome changes following exposure to drugs, chemicals, or irradiation probably play an important role in the development of AML (196,197). In secondary AML especially deletions of the long arm of chromosomes 5 and 7 are frequently observed (196,197). These and other karyotypic aberrations of AML will be discussed in Chapter 3.

1.3 BIOLOGY OF AML

Like other cancers, AML essentially is a genetic disease which results from alterations in the structure or expression of critical genes (153,154,203-206). Most probably, the malignant transformation requires a step wise series of events, the so-called multistep pathogenesis (205). In particular, derangements of genes which normally control growth and differentiation are thought to play a role in the development of AML (153,154,204-210). These concern proto-oncogenes, tumor suppressor genes, growth factor genes, and growth factor receptor genes (208,211). Various mechanisms, including point mutations, translocations, or deletions may alter the structure or expression of these genes (153,154,204,206). The finding of non-random chromosome abnormalities associated with AML (summarized in Chapter 3) has focused on the role of specific genes which reside nearby chromosomal breakpoints (204,212-214). Although many studies have suggested that proto-oncogenes or growth factor genes might be involved in AML, their precise role in leukemogenesis is still unclear (206-208). The major issue concerning the role of growth factors and proto-oncogenes is whether dysregulation of these genes play a central role in leukemogenesis or merely reflects an epiphenomenon. Expression of some cellular proto-oncogenes like, c-*myc*, c-*myb*, c-*fms*, c-*fes*, and c-*fos*, in AML patients have been found to be associated with either the proliferative activity or the differentiation stage of the AML cells (215,216). Interestingly, high expression of c-*myc* or c-*myb* may be associated with shorter remissions in AML patients (216). Activation of the N-*ras* oncogene does not depend on a chromosome translocation but can be caused by a single mutation (217-219). Such mutations have been demonstrated in 15%-30% of AML cases (219-221). Recent studies support evidence that activation of N-*ras* does not initiate AML, but contributes to the outgrowth of more malignant subclones (222).

Several investigators have found autonomous proliferation of AML cells *in vitro*, which finding provides further evidence that growth factors might play a role in the pathogenesis of AML (223-233). Autostimulation can be achieved by autocrine or paracrine growth factor production, altered growth factor receptor expression, or by internal autostimulation that bypasses membrane receptors (207,209,210,224). However, like their normal counterparts, most AML cells still require growth factors for *in vitro* growth and maturation (209). Interestingly, several investigators have found autocrine IL-1 (225-229) or TNF (230-233) synthesis in AML, which cytokines might induce production of other growth factor cytokines resulting in blast cell proliferation.

The leukemic cell population in AML is derived from a single malignantly transformed progenitor cell. In some patients the leukemic transformation occurs in a committed precursor cell, whereas in other patients involvement of pluripotent progenitor cells will result in AML involving various differentiation lineages (234-240). The transformed cell population is characterized by an increased self renewal potential and/or a decreased ability of differentiation. However, this differentiation and maturation block is usually not absolute, given the various maturation stages within an AML clone (156,157,161,241,242).

Furthermore, additional genetic derangements might occur, leading to subclone formation within an AML. The heterogeneity of AML between patients probably reflects, transformation in different stages of hematopoiesis as well as the differences in residual maturation abilities of the transformed cells.

The clonal origin of AML can be demonstrated by use of several techniques, like cytogenetics (204,212-214), determination of X-chromosome linked isoenzymes (243-245), analysis of X-chromosome linked DNA polymorphisms (246-248), and analysis of *ras* gene mutations (249). AML is not the only clonal disorder of myelopoiesis as clonality has also been demonstrated in aplastic anemia (AA), paroxysmal nocturnal hemoglobinuria (PNH), myeloid dysplastic syndrome (MDS), and chronic myeloproliferative disorders, such as chronic myeloid leukemia (CML) (250-256). Patients having one of these disorders are known to have an increased risk of AML development (251,252,255-259). Furthermore, MDS, AML, and some AA share comparable karyotypic abnormalities (213,214,252,257,260, 261). It has been hypothesized that clonal hematopoiesis represents an intermediate stage in the multistep pathogenesis of AML (262).

1.4 DIAGNOSIS OF AML

Introduction

The clinical manifestations of AML and other types of acute leukemias are directly or indirectly due to proliferation of leukemic cells and infiltration into normal tissues. Increased cell turn-over has metabolic consequences, e.g. hyperuricemia, hyperkalemia, and hyperphosphatemia, whereas the infiltrating cells disturb tissue function (1). As a consequence of BM infiltration anemia, neutropenia, and thrombocytopenia occur, which can lead to infection and hemorrhage. AML is heterogeneous with respect to biology, and as a result of the biologic heterogeneity, a wide variety of clinical presentations can be seen. In general, there is an acute onset of disease with malaise, fever, anemia, and hepatosplenomegaly. However, in some elderly patients a more gradual development of symptoms can be seen, especially if the AML is preceded by an MDS (263).

The purpose of disease classification is the clustering of diseases which share common features of etiology, pathogenesis, biology, clinical presentation, and response to therapy. Leukemia classification systems have traditionally been based on morphological criteria and different subvarieties of the disease are recognized according to the nature of the predominating cells (156,157). Recent advances in immunology, genetics, and molecular biology have enabled the characterization of additional features of acute leukemias, which may determine or reflect etiology, pathogenesis, or biology of the malignancy in a more direct way.

Morphological classification

The most widely used classification of acute leukemias is the French American British (FAB) classification, which was first published in 1976 and which was subsequently expanded, modified, and clarified (157-159,264-266). The FAB classification requires the examination of both PB and BM smears. The diagnosis is based on conventionally stained smears (e.g. Romanowsky staining or May Grünwald Giemsa staining) and a few essential cytochemical reactions, such as myeloperoxidase or Sudan black B and α-naphthylacetate esterase (156,157,267). Diagnostic criteria for acute leukemias such as established by the FAB group as well as the proposed system for classification of AML are outlined in Tables 3 and 4, respectively (156-158,264-266,268-270). The FAB classification of AML is essentially based on features of morphological maturation; in those acute leukemias which lack typical morphological characteristics classification might be difficult (156,159,160,265). More advanced techniques such as ultrastructural cytochemistry and immunological marker analysis have been incorporated in the FAB criteria for the diagnosis AML-M0 and AML-M7 (Table 4) (159,265,266,271-273). Additional enzym cytochemical reactions, such as specific esterase, periodic acid-Schiff reaction, and acid phosphatase may be applied to obtain insight in the cytoplasmic differentiation of the AML (156,160,267). The FAB group has defined criteria for the separation of AML from MDS as well as criteria for the classification of MDS (156,157,274). The prognostic value of the AML FAB classification is limited, although it has been reported that cases of AML-M5 and M6 have a slightly worse prognosis than those of AML-M1, M2, M3, and M4 (156,160,275). In most studies prognosis of AML-M0 and AML-M7 has been reported to be particularly bad (156,160,271,272, 275). A poor prognosis is also associated with the presence of AML with MDS features in all myeloid differentiation lineages (156,160,276-278). These examples demonstrate that maturation characteristics of AML at least partly reflect the biology of the malignancy. This can further be illustrated by the association between specific FAB subtypes and some distinct clinical features, such as disseminated intravascular coagulation (DIC), central nervous system (CNS) leukemia, skin infiltration, and myelofibrosis (Table 4).

TABLE 3. FAB criteria for acute leukemia diagnosis[a].

- At least 30% of the total nucleated cells in BM are blasts[b], or
- in the case of BM showing erythroid predominance (erythroblasts comprising ≥ 50% of total nucleated cells), at least 30% of non-erythroid cells are blasts[c], or
- if the characteristic morphological features of hypergranular promyelocytic leukemia (AML-M3 or M3 variant) are present.

a. Adapted from reference 156-158.
b. It has been recommended that occasional cases, who have less than 30% blasts in BM, but more than 30% myeloblasts in PB, should be diagnosed as having AML (268,269).
c. Lymphocytes, plasma cells, and macrophages also being excluded from the differential count of non-erythroid cells.

TABLE 4. Morphological classification of AML according to the FAB criteria[a].

FAB subtype	Leukemia type	Percentage of AML cases[b]	Typical clinical syndromes
M0[c]	myeloblastic without cytological maturation	5	
M1	myeloblastic with minimal maturation	15-20	
M2	myeloblastic with significant maturation	30-35	
M3	acute promyelocytic leukemia (APL)	5-10	DIC and bleeding diathesis
M3 variant	APL unusual hypogranular form		
M4	acute myelomonocytic leukemia (AMML)	15-25	CNS leukemia (M4Eo)
M4Eo	AMML with eosinophilic maturation		
M4Baso[d]	AMML with basophilic maturation		
M5a	monoblastic poorly differentiated	10-15	gumhyperthrophy, skin infiltration
M5b	monoblastic more differentiated		
M6	acute erythroid leukemia	4-6	
M7[e]	acute megakaryoblastic leukemia	1-4	myelofibrosis

a. Adapted from references 156-160,264-266.
b. According to references 156,160,270.
c. For diagnosis AML-M0 leukemic cells should be positive for at least one myeloid antigen, such as CD13 or CD33, and the leukemic cells should be negative for lymphoid antigens (CD2, CD4, and CD7 are not regarded as lymphoid markers). AML-M0 is often positive for myeloperoxidase demonstrated by immunocytochemistry and/or electron microscopy analysis (159).
d. Not described by the FAB group.
e. AML-M7 diagnosis should be confirmed by ultrastructural demonstration of platelet peroxidase or by (monoclonal) antibodies against platelet antigens (e.g. CD41, CD42, CD61, and factor VIII related antigen) (265,266).

Immunological classification

The hybridoma technology has permitted the generation of large series of monoclonal antibodies (McAb) against differentiation antigens expressed by leukocytes (279-282). In hematological malignancies, such as AML, McAb can be used for immunophenotyping of the tumor cells (283-286). The development of microscopic and flow cytometric techniques enabled the application of the so-called immunological marker analysis in diagnosis of AML (286,287). Immunological marker analysis can be used to characterize the various subpopulations present in heterogeneous AML cell populations. Furthermore, it is helpful, but not always conclusive, to differentiate whether a very immature type of acute leukemia represents an AML or ALL, which has important consequences for choosing the optimal therapy (159,160,271-273,288,289). Although immunological marker analysis is useful for the diagnosis of AML-M0, AML-M7, and immature types of AML-M6, in general their is only a partial correlation between immunophenotypes and the FAB types (159,290-293). The different immunological markers which can be used as well as the techniques to detect immunological markers are summarized in Chapter 2. Furthermore, in Chapter 2.2 data on the various applications of immunological marker analysis in the diagnosis of AML as well as the prognostic significance of the expression of certain antigens on AML cells will be discussed.

Cytogenetic classification

In the majority of AML patients clonal chromosomal aberrations can be found (212-214). The prognostic significance of the cytogenetic classification of AML has been demonstrated in different studies (212-214,270,294-296). As has been mentioned above, the finding of non-random chromosomal abnormalities in AML patients has yielded important information with regard to etiology and biology. Karyotypic abnormalities which are regularly found in AML patients are summarized in Chapter 3. For several chromosome abnormalities a strong correlation with a specific FAB type has been demonstrated (see Chapter 3.1). Other karyotypic abnormalities are associated with trilineage AML or MDS. A close association between genotype and phenotype in AML provides further evidence that the biology of the different types of AML are related to, or caused by, specific chromosome changes. Evidence for important links between molecular defects and morphological characteristics in AML comes from recently obtained results on the genes involved in the translocation t(15;17)(q22;q21) in AML-M3 (297-299). This translocation splices the retinoic acid receptor (RAR)-α gene on chromosome 17, which plays a significant role in cell differentiation. Important information on the relation between genotype and phenotype may come from extensive immunological marker analysis on AML with specific chromosome abnormalities. Data on the association of karyotypic abnormalities and specific immunophenotypes in AML are summarized in Chapter 3.

Other techniques to characterize AML

In addition to cytomorphology, immunological marker analysis, and cytogenetics other techniques can be used for the characterization of AML. These especially concern ultrastructural morphology, cell culture techniques, and recombinant DNA techniques.

Ultrastructural morphology and ultrastructural cytochemistry may be helpful in some specific cases, such as AML-M3, AML-M6, and, as has been mentioned above, AML-M0 and AML-M7 (300-303). The prognostic significance of electron microscopy (EM) is not clear, but most probably such analysis is only valuable if light microscopy is inconclusive (159,265,302).

Cell culture techniques are especially important in basic research of AML. There is marked patient to patient variation with regard to growth factor responsiveness *in vitro* and this heterogeneous reactivity appears not to correlate with the morphological classification of AML (304,305). In case of undifferentiated types of acute leukemias differentiation induction by use of short term *in vitro* cultures might be of diagnostic value (306). In addition, specific growth patterns *in vitro* may have prognostic significance for obtaining remission (305). Therefore, variations in growth factor responsiveness may provide another way of classifying AML patients with biologically different disease (307).

Recombinant DNA techniques have enabled analysis of genes at the DNA and mRNA

level (308). Specific probes can be used to detect gene rearrangements, mutations, or deletions. RNA studies enable structural analysis as well as quantitation of specific transcripts. The polymerase chain reaction allows the amplification of a specific piece of DNA or RNA, which fragments can be analyzed by use of other techniques (309). At this stage techniques of molecular genetics are a powerful research tool and probably they will be applied in AML diagnosis in the near future, especially in those cases in which other techniques are not conclusive (310-314).

EM, cell culture systems, and recombinant DNA techniques have in common that they are relatively expensive and time consuming and that the application of these techniques needs high technical skill and background information about structure and expression of genes during normal and abnormal differentiation.

Combined classification systems

During the last 5 years several attempts have been made to integrate various types of classification systems of acute leukemias in particular morphology, immunological marker analysis, and cytogenetics into a combined classification system (292,293,315-321). The morphologic, immunologic, and cytogenetic (MIC) working classification of AML established 10 AML subtypes, mainly based on unique combinations of cytogenetic aberrations and specific FAB subtypes (319). The MIC cooperative group identified a few specific immunophenotypes which showed some association with one of these 10 MIC subtypes. Furthermore, they emphazised to the value of immunological marker analysis to differentiate between ALL or AML in case of undifferentiated types of acute leukemia. The MIC group recognized the prognostic significance of specific karyotypic aberrations in AML (319). Large studies on the prognostic value of the MIC classification are still lacking. An alternative classification system of AML, which may have prognostic value, has been proposed by Hayhoe (318). He avoids to use the predominant cell population as primary, but instead divide AML into those with blast cell populations deriving from a committed clonogenic stem cell and those with origins from a pluripotential stem cell. Although primarily based on cytomorphology, this classification system does not exclude contributory features from immunological marker analysis, cytogenetics, or FAB classification.

1.5 PROGNOSIS AND TREATMENT OF AML

Introduction

Since the first application of chemotherapy in acute leukemia in the early 1950's and especially after the introduction of therapies using combinations of different drugs, progress has been made in the treatment of AML. In contrast to the high cure rates in childhood ALL,

the majority of AML patients still die of their disease (322-324). Clinically relevant adverse prognostic features include increasing age, a history of MDS, secondary AML, and certain cytogenetic abnormalities, especially those involving chromosomes 5 or 7 (263,276,277,294-296,325-328). All these features are probably associated with a defect in an immature precursor cell type (235).

In addition to conventional chemotherapy, bone marrow transplantation (BMT), and more recently, the application of growth factors and differentiation factors have become important treatment options for some patients (322,329-334). Furthermore, progress in supportive treatment with anti-microbial therapy and growth factors, which shorten the phase of critical neutropenia, has enabled a more successful infection prophylaxis and may allow a more aggressive approach in high risk AML patients (323,334).

Chemotherapy

The various drugs which are used in AML therapy are beyond the scope of this thesis and have been reviewed by others (322-324). In general, two phases of chemotherapy can be recognized, i.e. induction treatment and post remission treatment. Induction therapy will cause BM aplasia and should induce complete remission (CR). At present, induction with a combination of cytosine arabinoside (Ara-C), an anthracycline (usually daunorubicin), and one or two other drugs achieves CR in 55% to 80% of AML patients (322-324). On the other hand, about one third of all patients do not obtain CR and most of these patients will die within 2 months after diagnosis. This especially concerns patients over the age of 60 years, at which ages the highest incidence of AML occurs (322,323) (Figure 2). Despite leukemic cell kill in those patients who obtained CR, a considerable number of AML cells (probably 10^7 to 10^9) will remain in the majority of patients after induction treatment (322,335). Such numbers of tumor cells are usually undetectable by standard cytomorphology of BM and PB samples.

Post remission treatment attempts to eradicate remaining AML cells. Several strategies of post remission therapy have been proposed, such as double induction, intensive consolidation, and long term maintenance treatment (322-324,335). There is no general agreement about the optimal intensity and duration of post remission chemotherapy (322-324,335-339). For elderly over 65 years of age a less toxic therapy using low dose Ara-C has been proposed (340,341). However, in a selected group of elderly patients with a good performance status full intensity induction and post remission treatment can achieve long term results similar to younger patients (341,342).

The median remission duration ranges from about 10 to 20 months in most studies (322-324,335). The reported 5-year disease free survival rates range from 10% to 25% in adults and up to about 50% in children (322-324,336,337,343). Comparison of studies on the therapy of such a heterogeneous disorder as AML should be performed cautiously because of differences in patients groups and selection of controls and because the

number of patients in follow-up is often too low (344). Moreover, comparison of results may be hampered by the fact that some studies on post remission therapy, such as post remission chemotherapy and BMT, are applied only on those patients who have entered CR (344). Therefore, survival rates from studies on post remission chemotherapy are higher, ranging from 15% in some studies in elderly patients to 65% in a German study on AML in children (322,343,345).

Sometimes treatment of acute leukemia fails because of drug resistance, which may be caused by a mutation or an increase in expression of a so-called multidrug resistance (MDR) gene (346-348). This results in overexpression of a 170 kDa membrane glycoprotein (P-glycoprotein) which functions as an energy dependent membrane pump (347). Alterations in the expression of the MDR gene can be demonstrated at the RNA level or by use of a McAb against P-glycoprotein (346-348). The effectiveness of chemotherapy can be enhanced by using different drugs which are not cross-resistant, dose escalation of the drugs, or specific pharmacologic agents which are able to block drug resistance (e.g. verapamil, cyclosporine, and tamoxifen) (347,349,350).

Bone marrow transplantation

For patients who have entered CR BMT enables a myeloablative therapy, which is the most aggressive form of post remission therapy (322,329,330). In case of allogeneic BMT the patient is rescued with BM provided by an HLA identical sibling or, more recently, by a transplant from an unrelated HLA compatible donor (351). In autologous BMT (ABMT) patient's own BM and/or peripheral stem cells are taken and stored before total body irradiation and/or the administration of high dose chemotherapy (352).

Allogeneic BMT is generally performed in patients up to the age of ~55 years, because peritransplant mortality increases with age (322,329,330). For patients transplanted in first CR with BM from an HLA identical sibling 5 years survival rates ranges from 40% to 60% (322,329,330). Despite the good results of some recent studies of post remission chemotherapy, it has been reported that results of allogeneic BMT are equivalent or superior (322,329,330,353-355). Therefore, allogeneic BMT is recommended for young AML patients in first CR who have an HLA identical sibling. It should be mentioned that, due to fact that the median age of AML patients is 60 years as well as the lack of donors in most cases, less than 10% of all AML patients turn out to be candidates for allogeneic BMT (322,329,356). Comparison of T cell depleted allogeneic BMT and allogeneic BMT without T cell depletion has demonstrated the essential role of T lymphocytes in BMT. Although T cells may mediate graft versus host (GVH) reactivity, they also facilitate engraftment of the transplant (322,357,358). In addition, the efficacy of allogeneic BMT depends among others on a T cell mediated graft versus leukemia activity (322,329,330,359).

In ABMT the risk of GVH reactivity is not present. Therefore, ABMT is less toxic than allogeneic BMT, but it is also less effective, probably by the absence of the graft versus

leukemia activity (322,360,361). Furthermore, there is a risk of reinfusion of clonogenic AML cells. The autologous BM graft can be treated *in vitro* by use of various techniques to remove residual AML cells (352). Controversy exists about the importance of these so-called purging procedures and the role of residual leukemic cells in the autologous BM transplant (322,352,362-365). The differences in protocols used for ABMT hamper a proper evaluation of its value in AML treatment. There is often a significant delay from achievement of CR to transplantation, which causes selection of patients who may already be cured (352). An extended follow-up is needed to demonstrate the value of the various post remission therapies.

Other treatment options in AML

It has been suggested that more intensive chemotherapy than performed at present does not cure more patients (366,367). Therefore, other strategies of treatment are needed to obtain higher cure rates. The role of T lymphocytes in the process of graft versus leukemia in allogeneic BMT has initiated research on the possibilities to use T cell immunotherapy in AML (359,368,369). For this approach autologous T cells might be activated by biological substances such as IL-2 (370,371). Alternatively, sequential increments of allogeneic T cells, either *in vitro* activated or not, might be administrated to the patient at different time intervals (372). Preclinical data on these types of post remission treatment are promising (371,372).

It has been mentioned above that the transformed cell population in AML is characterized by an increased self renewal potential and a decreased ability of differentiation. Several attempts have been made to induce terminal differentiation in AML and a few chemotherapeutic agents are able to cause some differentiation, e.g. low dose Ara-C (340). As has been mentioned previously, in AML-M3 with t(15;17)(q22;q21) critical elements of the genes coding for the RAR-α are replaced as a consequence of the chromosomal translocation (297-299). It has been found that all-*trans*-retinoic acid is extremely successful in inducing differentiation of the leukemia cells in patients with an AML-M3 (373-376). Such treatment can result in CR and prevent DIC, but chemotherapy remains necessary. Growth factors, such as granulocyte macrophage colony stimulating factor (GM-CSF), may be used to induce differentiation and proliferation of AML cells *in vivo*, which might increase the efficacy of some chemotherapeutic drugs (377-378). However, results of a recent study suggest caution in the use of GM-CSF because it may increase resistance of AML cells to chemotherapy (379).

Although AML is a heterogeneous disorder, treatment is rather uniform. The exciting developments in the treatment of AML-M3 demonstrate that patients with certain well defined subtypes of AML might benefit from a more specific therapy. In the near future it might be expected that several specific genetic defects will be identified, which allow the development of specific therapeutic modalities designed to correct or antagonize these

defects and improve the change of curing AML.

1.6 SCOPE OF THE THESIS

The heterogeneity of AML is subject of this thesis. The purpose of this study was to characterize the different subpopulations which can be found in the various types of AML at diagnosis and during follow-up. For this purpose we especially used extensive immunological marker analysis. In close collaboration with clinical scientists and laboratory scientists our results were correlated with other features of these patients, especially clinical characteristics and cytogenetics. Both the clinical significance and some basic aspects of specific immunophenotypes in AML patients were determined.

Chapter 2 summarizes characteristics of immunological markers, which are generally used for immunophenotyping of various differentiation stages of hematopoietic cells. Microscopic techniques and flow cytometric techniques, which can both be used for the detection of expression of these markers, are described. Data on marker expression in AML are summarized and their significance will be discussed.

Chapter 3 describes the results of immunological marker analysis in AML with specific non-random chromosome aberrations. Literature data on the association between specific karyotypic abnormalities and phenotypical features are summarized. We investigated the immunophenotypes of the various subpopulations which occur in AML with t(6;9)(p23;q34) and AML with inv16(p13q22). In both AML types we found a specific immunophenotype. The clinical and biological signifance of these results are discussed.

Chapter 4 is a compilation of four studies on the presence and significance of AML subpopulations expressing terminal deoxynucleotidyl transferase (TdT). TdT is normally expressed in immature lymphoid cells and is probably involved in the rearrangement processes of the genes which code for the antigen specific receptors of lymphocytes, the immunoglobulin (Ig) and T cell receptor (TcR) molecules. We investigated the occurrence of TdT^+ subpopulations in AML patients and in healthy control subjects. Furthermore, by use of double immunofluorescence (IF) staining, we determined the immunophenotype of these TdT^+ cells. We found that TdT is expressed in immature leukemic subpopulations which occur in the majority of AML patients, whereas TdT^+ myeloid cells are extremely rare or not detectable in healthy controls. Based on these results we started a follow-up study of AML patients to investigate whether TdT^+ AML subpopulations can be monitored during and after treatment. We found that double IF labeling for myeloid markers and TdT enables the detection of minimal residual disease. The clinical significance of these findings are discussed. Finally, we investigated whether rearrangements of Ig or TcR genes did occur in AML and whether these were related to TdT expression.

Chapter 5 discusses the significance of the presented experimental data and their clinical application in the context of the literature. In addition, we discuss some perspectives of the role of immunological marker analysis in AML characterization and clinical diagnosis.

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CHAPTER 2

IMMUNOLOGICAL MARKER ANALYSIS

CHAPTER 2.1

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

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INTRODUCTION

The characterization of cells in the various hematopoietic differentiation stages can be performed morphologically (1-5). Additional characterization is obtained by immunological marker analysis (6-12). The expression of a particular set of immunological 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 immunological markers, how the expression of these markers can be detected and how immunological markers can be used for the characterization of normal hematopoietic cells as well as leukemic and NHL cells.

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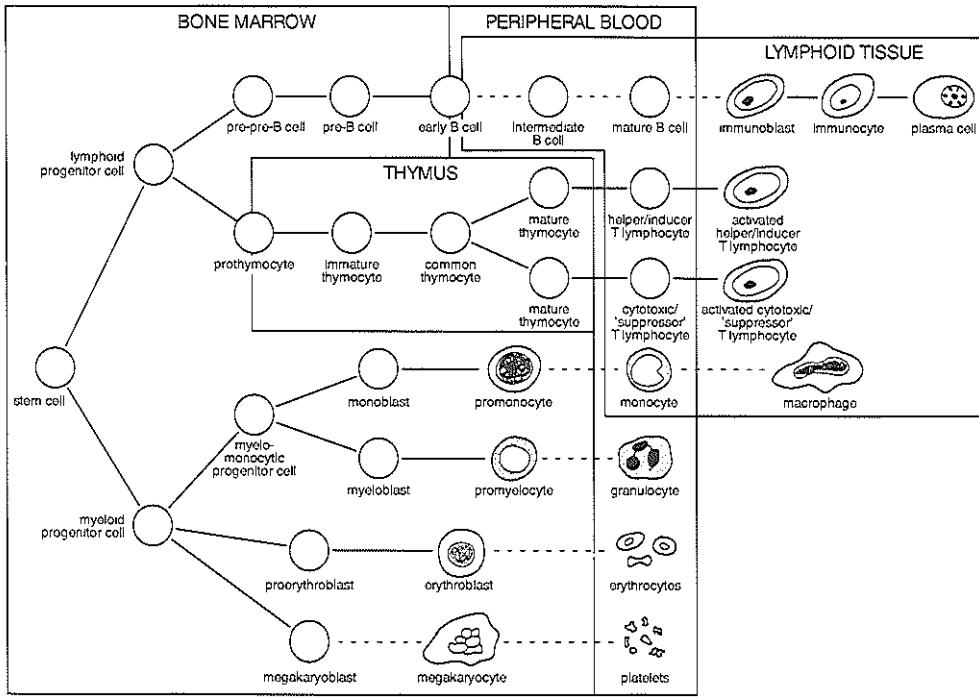

Figure 1. Hypothetical scheme of hematopoiesis. The different compartments, where cells in the various differentiation stages are located, are indicated.

HEMATOPOIETIC DIFFERENTIATION STAGES AND IMMUNOLOGICAL MARKERS

Hematopoietic differentiation can be divided into a lymphoid and a myeloid lineage. The 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 immunological markers (6-12,19-24). Although immunological 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 immunological markers per differentiation stage is summarized in the Figures 3 and 4. As is illustrated by these figures, markers for the lymphoid differentiation

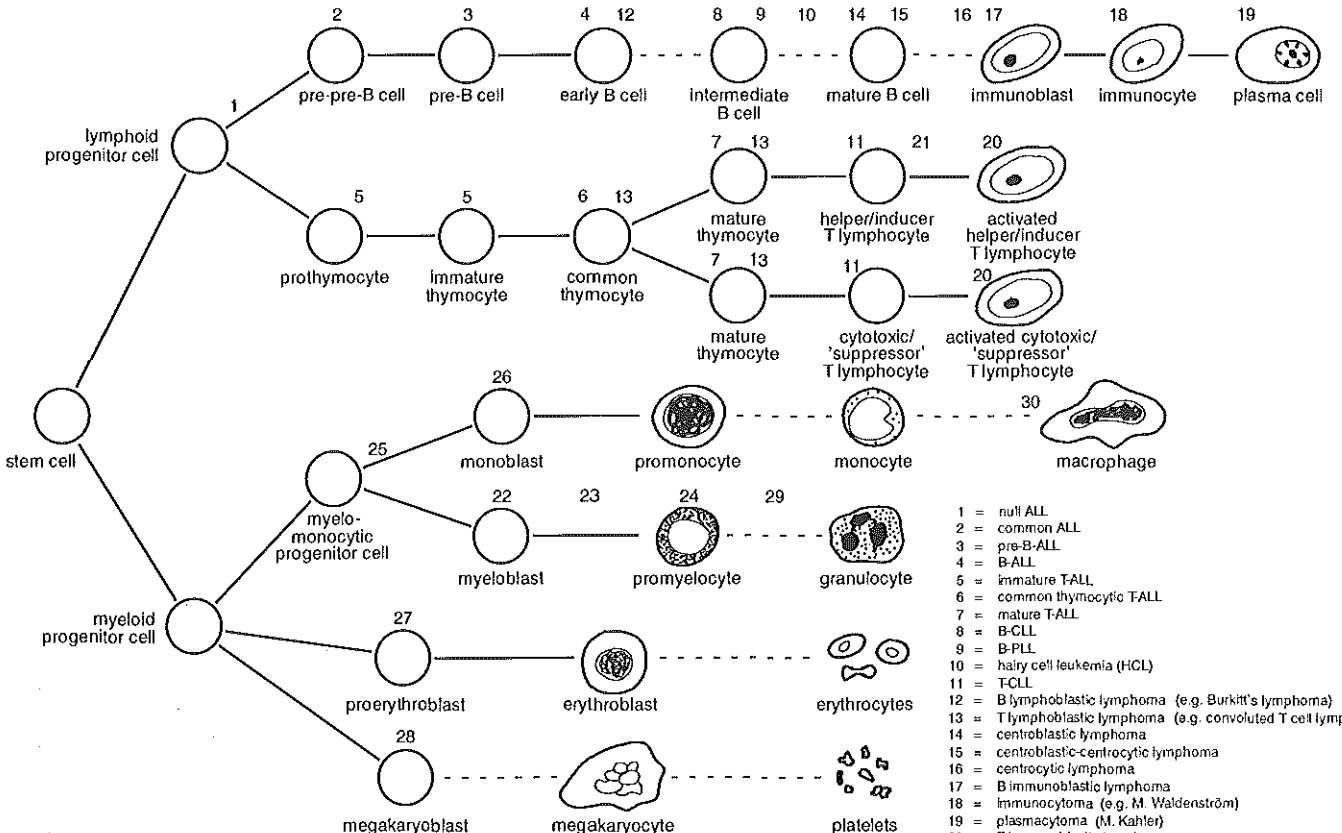

Figure 2. Hypothetical scheme of hematopoiesis. The numbers indicate as to where the various leukemias and NHL can be located according to their maturation arrest (see insert). The nomenclature of the NHL, indicated by numbers 12 to 20 is derived from the Kiel classification of NHL (4). The numbers 22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

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

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

International nomenclature and classification of immunological markers

In order to classify the large number of monoclonal antibodies (McAb) against immunological 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 immunological 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). In the following sections the various McAb and the recognized markers will be indicated by their CD codes. When specific immunological 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).

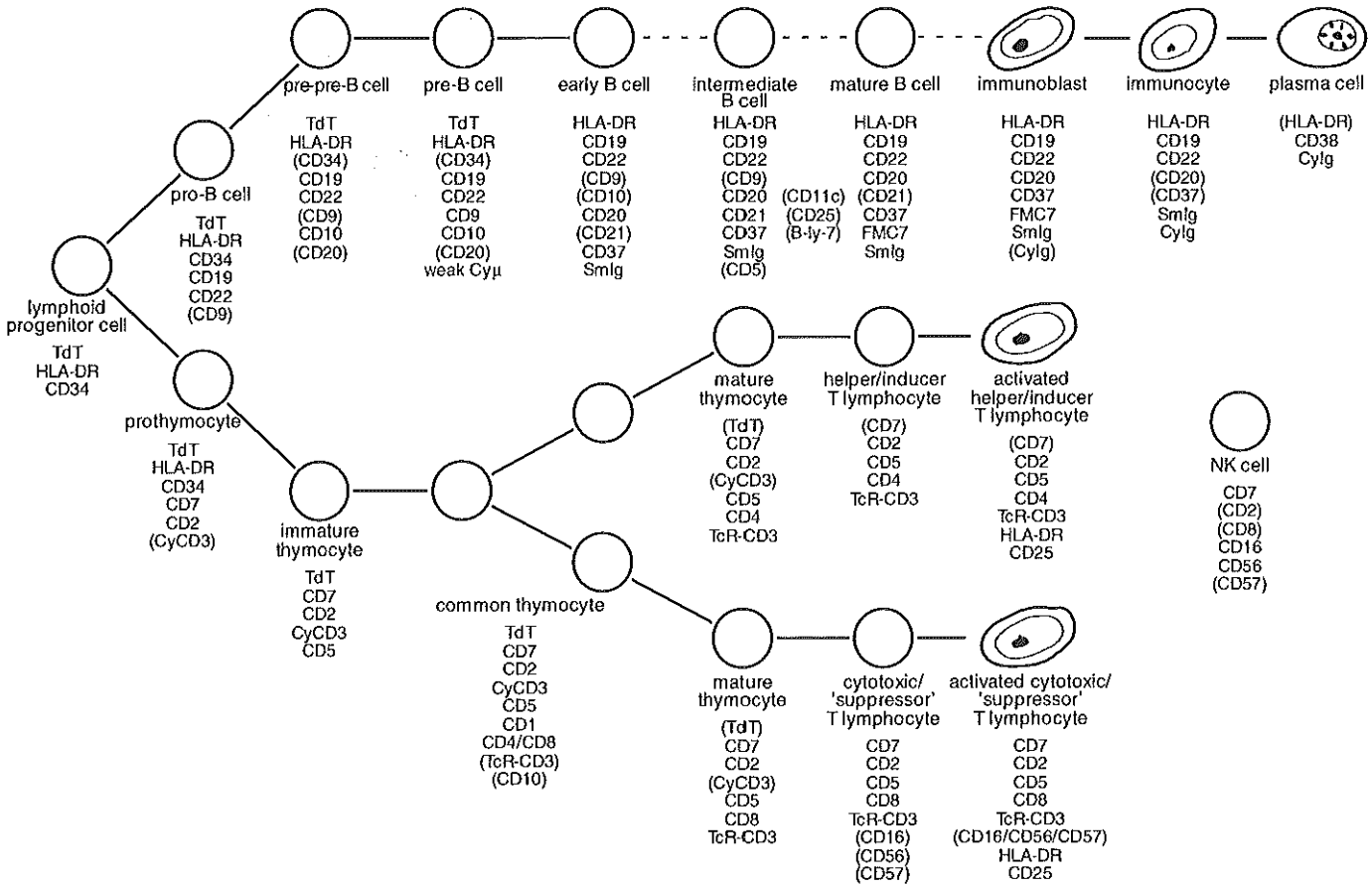

Figure 3. Hypothetical scheme of lymphoid differentiation. The expression of the various immunological markers by cells in the different stages of lymphoid differentiation are indicated. The markers in parentheses are not always expressed.

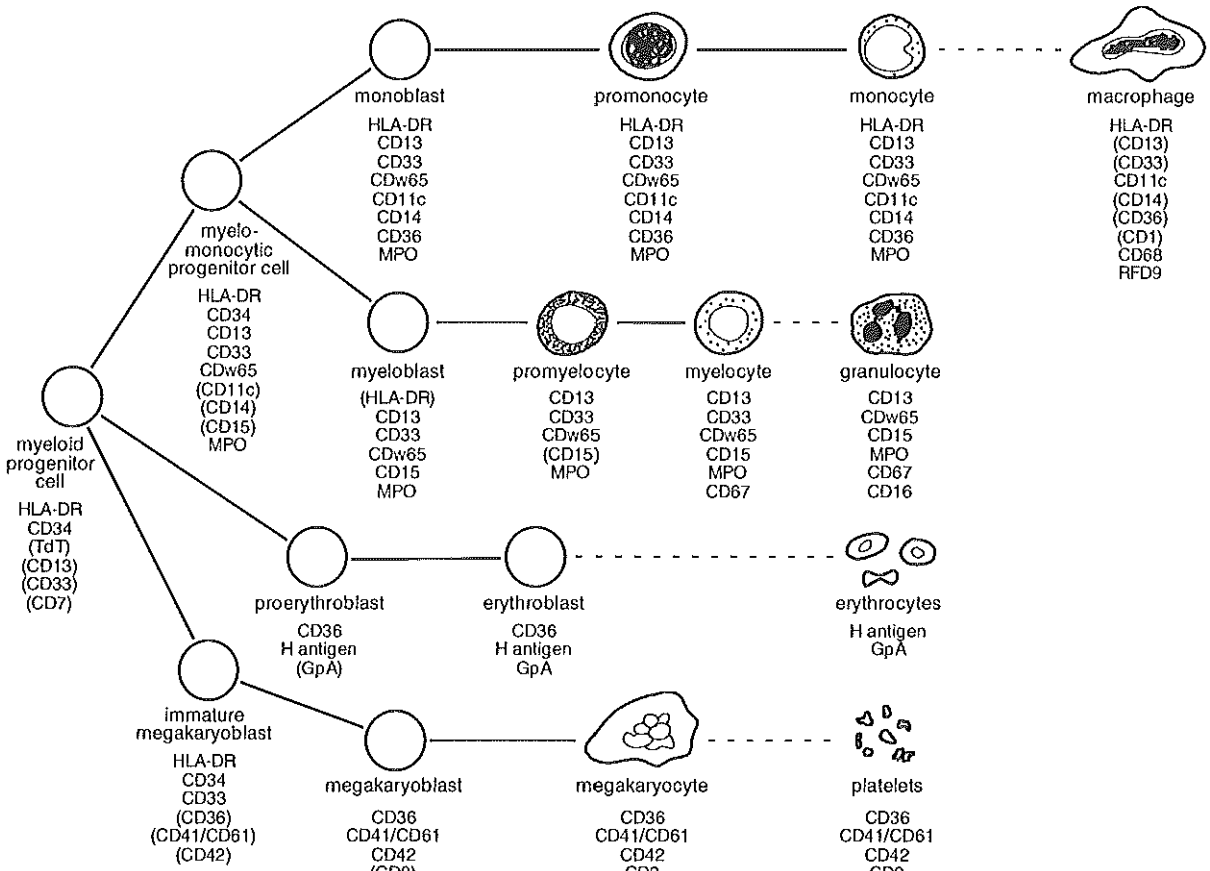

Figure 4. Hypothetical scheme of myeloid differentiation. The expression of the various immunological markers by cells in the different stages of myeloid differentiation are indicated. The markers in parentheses are not always expressed.

TABLE 1. Immunological 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[l,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], 6BIIC7[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[n], 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[j]	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
CD64	32.2[y]	high affinity Fc receptor for IgG (FcγRI) (gp75)	monocytes	18,84
CD68	Ki-M6[d], Ki-M7[d]	macrophage antigen (gp110)	macrophages (Figure 4)	93
—	Monocyte-2/61D3[z]	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[a], 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, complement (C3bi, C3dγ) receptor (CR4)	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	113,114

CD[a,b] code	Antibodies[b]/ techniques	Antigen recognized	Reactivity with hematopoietic cells	References
CD23	Leu-20[h]	low affinity Fc receptor for IgE (FcεRII) (gp45)	subpopulation of B cells, monocytes, eosinophils	17,18,84
CD24	BA-1[q]	B cell-granulocytic antigens (gp42)	subpopulations of (precursor) B cells, granulocytes	39-41,124
CD25	2A3[h]	α chain of interleukin 2 receptor (IL-2R) (gp55)	activated T cells, activated B cells (e.g. HCL) (Figure 3)	17,18
CDw32	IV.3[y]	intermediate affinity Fc receptor for IgG (FcγRII) (gp40)	monocytes, subpopulation of macrophages, B cells, granulocytes, eosinophils	17,18,84
CD34	My10[h], BI-3CS[l]	precursor antigen (gp115)	precursors of lymphoid and myeloid cells cells (Figures 3 and 4)	17,18,111
CD36	OKM5[q], ESIVC7[x]	thrombospondin receptor (platelet GPIV) (gp90)	monocytes, macrophages, early erythroid cells, megakaryocytes, platelets	17,18
CD38	Leu-17[h], OKT10[q,r]	T10 antigen (gp45)	activated T and B cells precursor cells, subpopulations of B cells, plasma cells	17,18,41,108
CD54	Leu-54/LB-2[h],	intercellular adhesion molecule-1 (ICAM-1); ligand for CD11a-CD18 (LFA-1) (gp90)	monocytes, lymphocytes	18
CD55	BRIC110[aa]	decay accelerating factor (DAF) (gp70)	leukocytes, erythrocytes, platelets	18
CD58	G26[d], TS2/9[bb]	LFA-3 antigen; ligand for CD2 (LFA-2) (gp40-65)	leukocytes, erythrocytes	18
CD59	YTH53.1[cc]	Ly-6 antigen (gp18-20)	leukocytes, erythrocytes, platelets	18
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, France, 1982; Boston, MA, USA, 1984; Oxford, UK, 1986; Vienna, Austria, 1989).
b. Only McAb are included, which are routinely used in the immunodiagnostic laboratory of the Department of Immunology, University Hospital Dijkzigt, Rotterdam, The Netherlands.
c. Supertechs, Bethesda, MD, USA.
d. Behring, Marburg, Germany.
e. Dr. W. Knapp and Dr. O. Majdic, Vienna, Austria.
f. Coulter Clone, Hialeah, FL, USA.
g. Hybritech, San Diego, CA, USA.
h. Becton Dickinson, San Jose, CA, USA.
i. SeraLab, Crawley Down, UK.
k. Boehringer Mannheim, Mannheim, Germany.
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, USA.
r. American Type Culture Collection, Rockville, MD, USA.
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, USA.
w. Nichirei Co., Tokyo, Japan.
x. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; in other countries: Janssen Biochimica, Beerse, Belgium.
y. Medarex Inc., West Lebanon, NH, USA.
z. Bethesda Research Laboratories, Gaithersburg, MD, USA.
aa. Dr. D. Anstee, Bristol, UK.
bb. Dr. T. Springer, Boston, MA, USA.
cc. Dr. H. Waldmann, Cambridge, UK.

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 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 IMMUNOLOGICAL MARKERS OF CELLS IN SUSPENSION AND CELLS IN TISSUE SECTIONS

In recent years McAb have increasingly been used for the detection of immunological 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

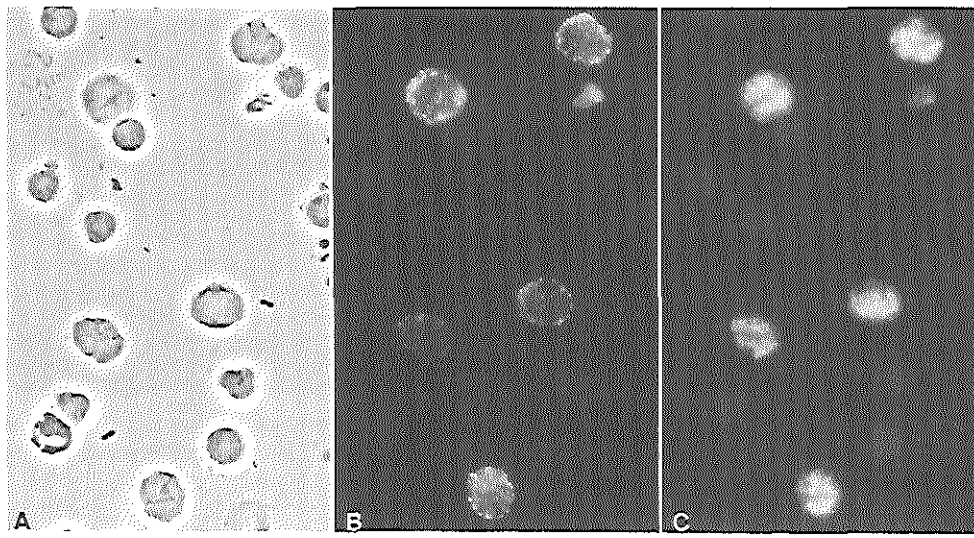

Figure 5. Double immunological staining for CD10 and TdT on MNC of PB from a patient in early relapse of an ALL. **A:** phase contrast picture; B: CD10 positive cells (TRITC labeled); **C:** TdT positive cells (FITC labeled). All TdT positive cells in the PB sample appeared to express CD10, indicating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

detection of immunological markers on hematopoietic cells in suspension (Figures 5-12) as well as on cells in tissue sections.

Immunological 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 antibodies. 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 or phycoerythrin (PE) (128,129). Although PE has advantages for fluorescence activated cell sorting procedures, we prefer to use TRITC in fluorescence

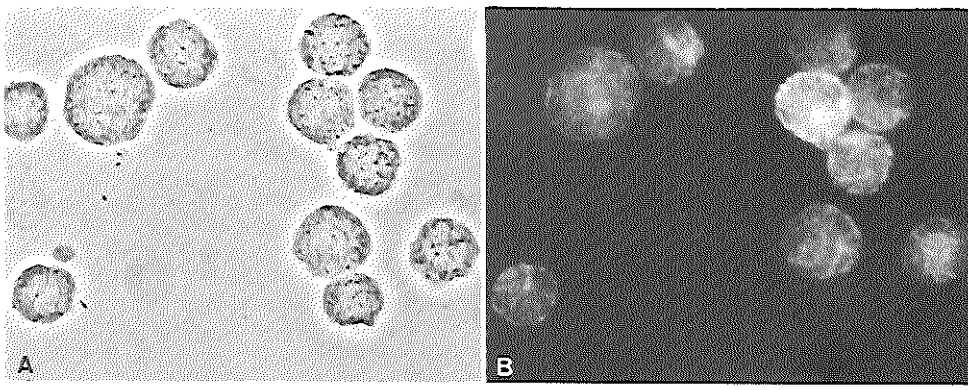

Figure 6. Immunological staining for cytoplasmic μ Ig heavy chain on MNC of BM from an ALL patient at diagnosis. **A:** phase contrast picture; **B:** weak Cyμ positive cells (FITC labeled). Most MNC of BM appeared to be weakly positive for Cyμ, indicating that it concerned a pre-B-ALL.

microscopy, because PE fades very quickly. If necessary, even a triple immunological staining can be performed using colloidal gold particles as the third label next to FITC and TRITC (Figure 12) (125). Such double and triple immunological 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 immunological 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.

Immunological 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 immunological 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

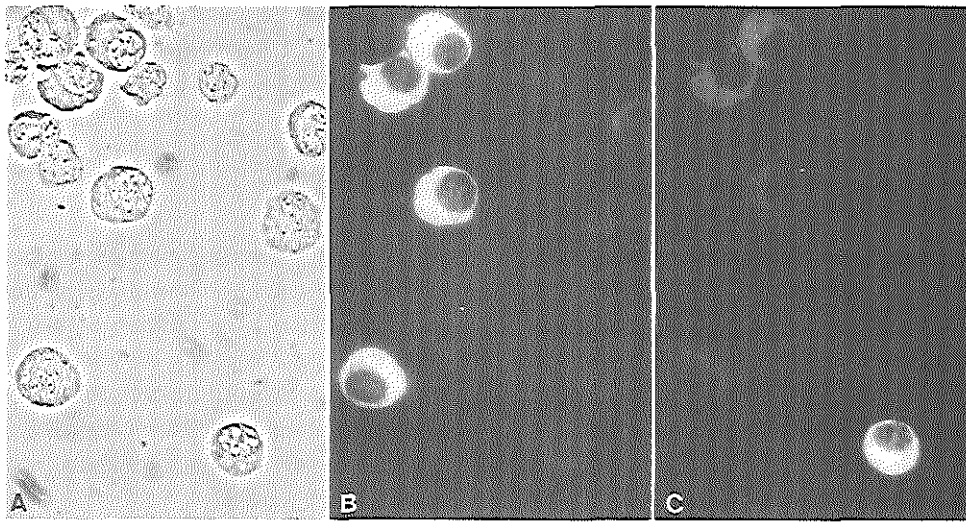

Figure 7. Double immunological staining for Cyκ and Cyλ on BM cells from a patient with a plasmacytoma. **A:** phase contrast picture; B: Cyκ positive cells (TRITC labeled); **C:** Cyλ positive cell (FITC labeled). The majority of the Cylg positive cells appeared to be positive for Cyκ; only a few Cyλ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

concerning methods for immunological 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 immunological markers (20). Nevertheless it may be necessary that a cell suspension is made from a tissue biopsy in order to perform immunological marker analysis, especially if a part of the cell suspension is used for cytogenetic analysis.

METHODS FOR IMMUNOLOGICAL 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, 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 immunological stainings

Most immunological 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 immunological 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 (IF) staining methods as well as the microscopes, which we use for the evaluation of the IF stainings.

Single immunological 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 immunological staining (e.g. using FITC-conjugated anti-human immunoglobulin (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 immunological 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, 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-aminoethylisothiouronium 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 x 10^6 cells per ml) is mixed with 100 µl AET treated SRBC (2 x 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 counting chamber and evaluated by combining epi-illumination (using the FITC filter combina-

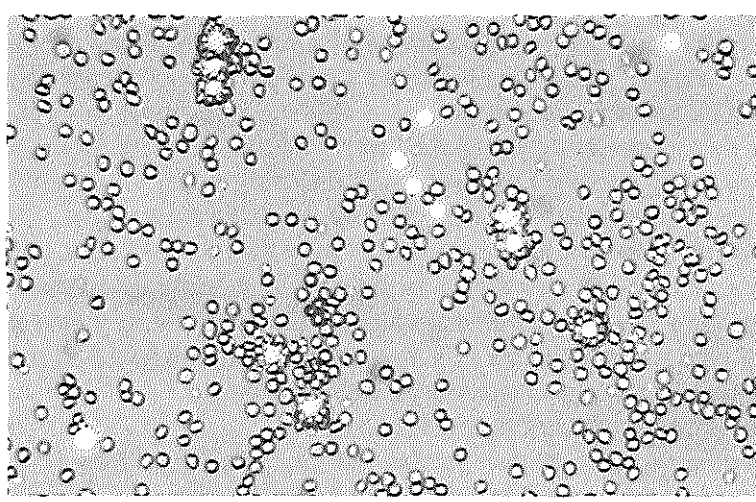

Figure 8. E rosette test on MNC of PB from a healthy adult. The nucleated cells were stained with acridine orange and evaluated by combining epi-illumination (using a FITC filter combination) with trans-illumination. Eight cells in the presented field form rosettes, while six cells do not.

tion) 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 immunological stainings

All of the various single immunological stainings, described above, can be combined. When antibodies are used in double immunological 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.
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 (NMS) 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 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.

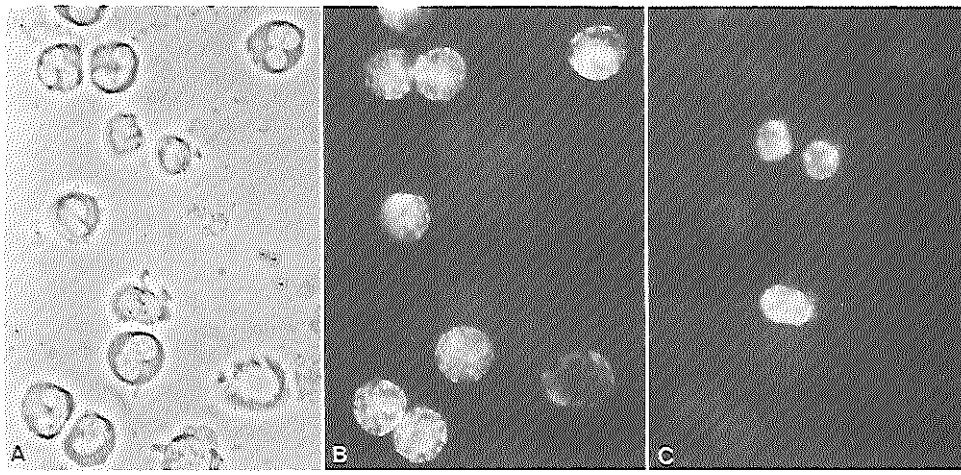

Figure 9. Double immunological staining for the CD15 antigen and TdT on MNC of BM from a CML patient in early lymphoid BC. **A:** phase contrast picture; **B:** CD15 (VIM-D5) positive cells (TRITC labeled); **C:** TdT positive cells (FITC labeled). The three TdT positive cells do not express the myeloid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

By the use of these four possibilities or their combinations, many different double IF stainings can be performed. Additional possibilities for double immunological 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 immunological stainings will be described.

Double IF 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 IF 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).

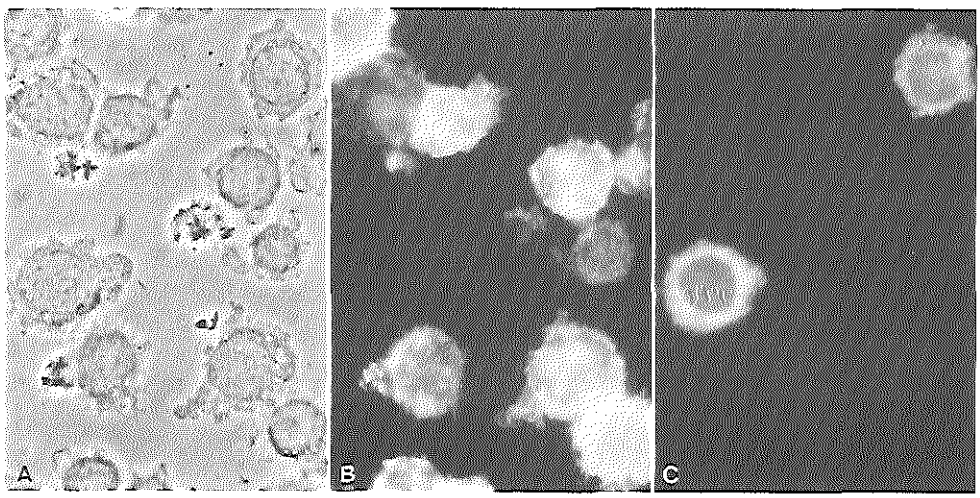

Figure 10. Double immunological staining for HLA-DR and Cyμ on cells of a Burkitt lymphoma derived cell line. **A:** phase contrast picture; **B:** HLA-DR positive cells (TRITC labeled); **C:** Cyμ positive cells (FITC labeled). The HLA-DR positive cells did not express Cyμ.

Double IF 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 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 IF 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 immunological 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).

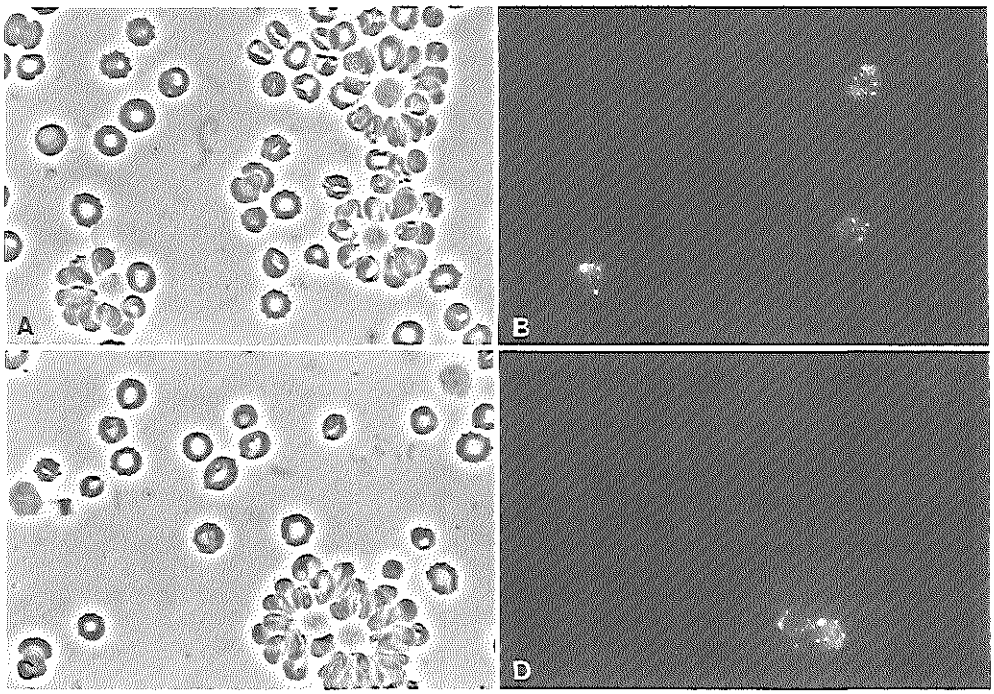

Figure 11. Double immunological staining for Smλ and M rosette on MNC of PB from a CLL patient. **A** and **C**: phase contrast pictures; **B** and **D**: Smλ positive cells (FITC labeled). A and B represent the same field and C and D represent the same field. The M rosette forming cells also weakly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

Triple immunological staining

In the triple immunological 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 system, which is used for the evaluation of fluorescence. 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 immunological staining will be described.

Triple immunological 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 NMS (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-

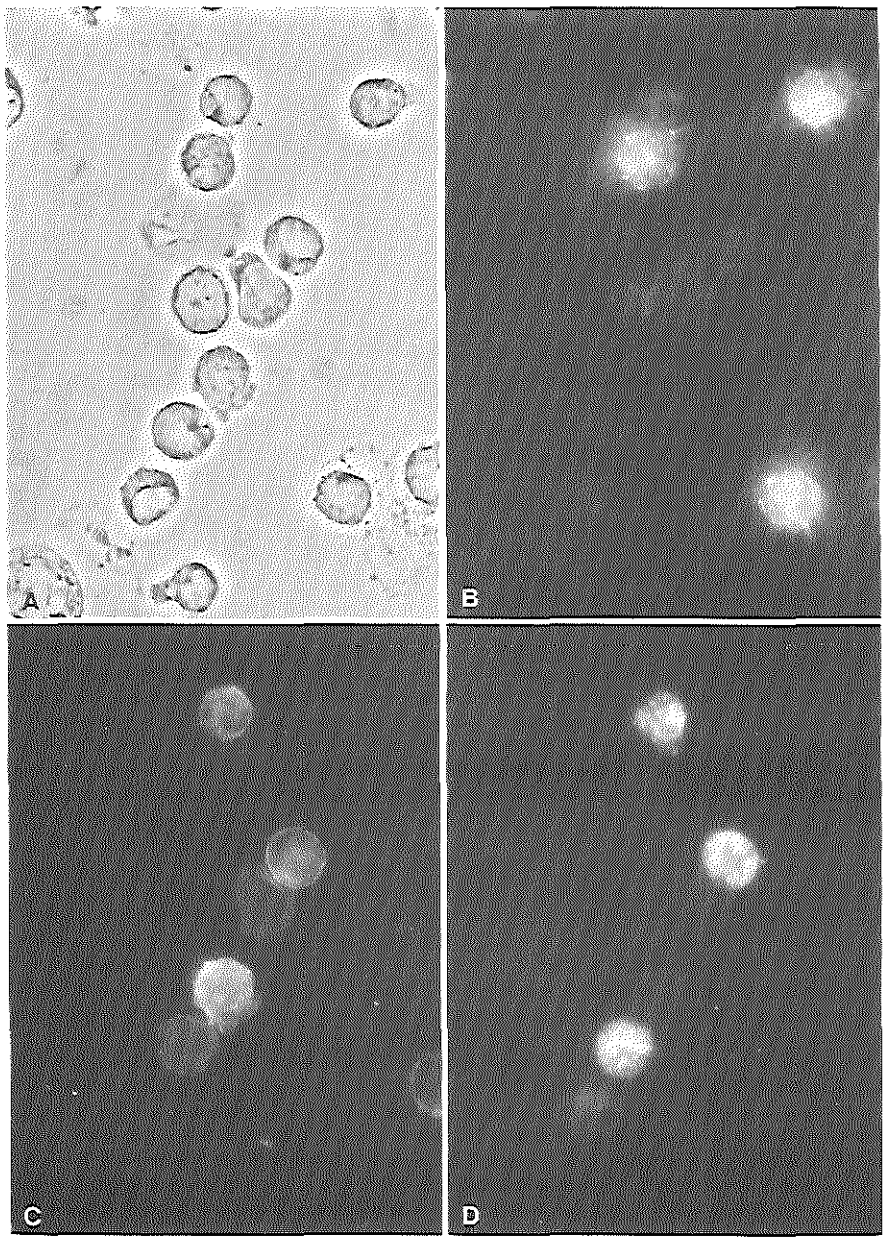

Figure 12. Triple immunological staining for the CD3 antigen, HLA-DR and TdT on MNC of BM from a child with ALL in complete remission under maintenance treatment. **A**: phase contrast picture; **B**: CD3 (Leu-4) positive cells (colloidal gold labeled); **C**: HLA-DR positive cells (TRITC labeled); **D**: 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.

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).

Microscopes

Evaluation of single and double immunological stainings, using fluorochromes.

For the evaluation of fluorescence (FITC, TRITC and acridine orange), we use Zeiss Standard 16 microscopes (Carl Zeiss, Oberkochen, Germany), 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 immunological 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, Germany). 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).

TABLE 2. Immunological phenotypes of ALL

	TdT	HLA-DR	CD19 and/ or CD22	CD10 (CALLA)	weak Cyμ	Smlg	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).

IMMUNOLOGICAL PHENOTYPES OF LEUKEMIAS AND NHL

Immunological 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 immunological phenotype.

Several differentiation stages may occur within one malignancy. This indicates that malignant 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 immunological phenotype: null ALL, common ALL, pre-B-ALL, B-ALL, and T-ALL (6,7,19,22,23,142). These immunological 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 immunological classification. L1 and L2 morphology can be found in most immunological 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 expres-

TABLE 3. Immunological 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.

sed. 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 immunological phenotypes of B-CLL, HCL, and B-PLL are summarized in Table 3.

Immunological marker analysis of NHL does not result in a classification comparable to the detailed morphological classifications such as the Kiel classification (4,148). However, immunological marker analysis is still valuable in the diagnosis of NHL (148). It is possible to 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 immunological markers (especially B cell markers) have become available, which enable a better immunological 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 immunological 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 immunological 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 immunological marker analysis of AML (24). Only double IF stainings for the various myeloid markers allow the immunological 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), immunological marker analysis can reveal whether it concerns a myeloid or lymphoid BC (Figure 9). The immunological 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 IMMUNOLOGICAL MARKER ANALYSIS

Immunological marker analysis of cells in normal hematopoietic differentiation stages

Immunological 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 disturbed (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 treatment) (157,158) (Table 4).

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

TABLE 4. Application of immunological marker analysis.

A. Immunological 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. Immunological 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 immunological 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.

Immunological marker analysis of leukemias and NHL

Immunological marker analysis of leukemias and NHL enables a reproducible classification of these malignancies. By means of clinical trials it may be possible to recognize immunological 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 immunological criteria, allowing adaptation of treatment protocols. Furthermore, immunological marker analysis is important for the detection of associations between immunological phenotypes and chromosome aberrations, to gain more insight in oncogenesis (160,161). In addition immunological 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 immunological 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 immunological detection of low numbers of malignant cells is the double IF 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).

CONCLUSION

Immunological 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.

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CHAPTER 2.2

IMMUNOLOGICAL MARKER ANALYSIS OF MYELOID DISORDERS, WITH SPECIAL EMPHASIS ON FLOW CYTOMETRIC TECHNIQUES

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INTRODUCTION

As has been described in Chapter 2.1 immunological marker analysis is useful for the characterization of normal hematopoietic cells as well as their malignant counterparts, i.e. the leukemias and the non-Hodgkin lymphomas (NHL). The visualization of the reactivity of the antibodies can be performed by use of fluorescence methods, which enable the performance of double or triple stainings. The evaluation of immunofluorescence (IF) stained cells can be performed with microscopic or flow cytometric techniques. The former technique has been described in Chapter 2.1. In the present chapter we shall discuss flow cytometry and summarize the applications of this technique in clinical diagnosis. Especially, the application of flow cytometry in immunological marker analysis of myeloid cells and myeloid disorders is described. At the end of this chapter the possibilities and limitations of both fluorescence microscopy and flow cytometry in immunological marker analysis are discussed.

FLUORESCENCE ACTIVATED FLOW CYTOMETRY

Structure of flow cytometer

During the last three decades progress has been made in the technology of flow cytometry as well as in computer technology to acquire and process a large amount of data (1-4). These developments have resulted in current "third generation" fluorescence activated flow cytometers for clinical practice. These instruments, e.g. FACScan (Becton Dickinson, San Jose, CA, USA), EPICS Profile II (Coulter Electronics, Hialeah, FL, USA), or CYTORON ABSOLUTE (Ortho Diagnostic Systems, Raritan, NJ, USA), are exclusively developed to analyze cell samples and lack cell sorter capabilities. A schematic diagram of a fluorescence activated flow cytometer is given in Figure 1. A typical instrument consists of an air cooled

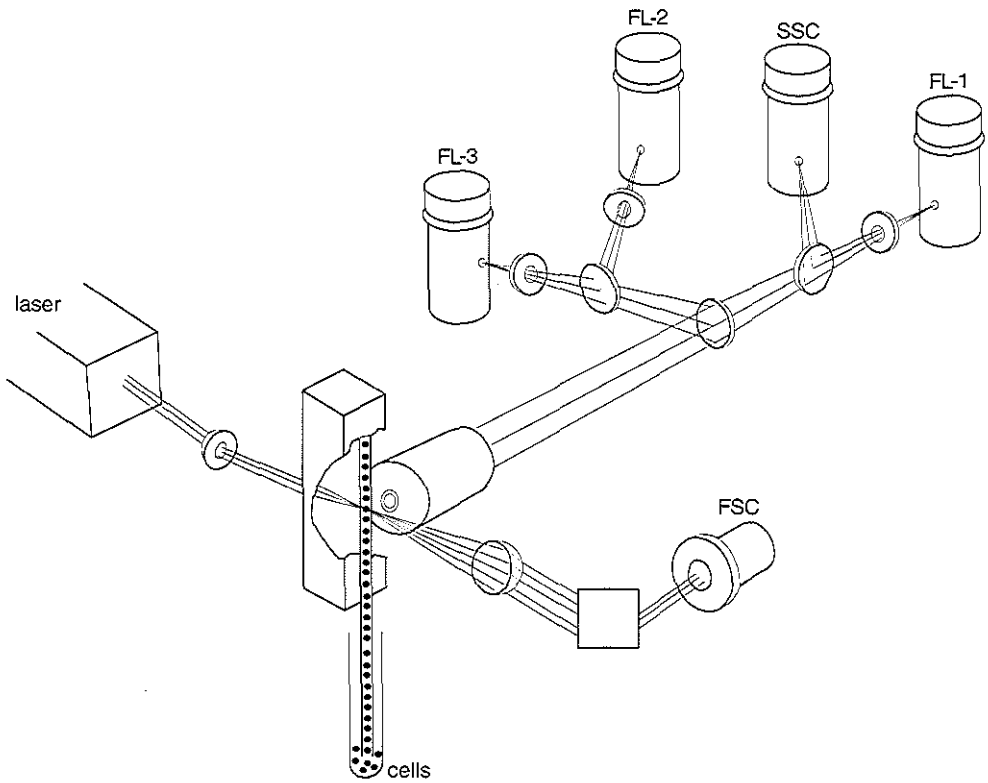

Figure 1. Schematic diagram of a flow cytometer. Laser beam is focused on the cells passing through the flow chamber. Refracted light is measured at a low angle in foward direction (FSC signal) and at 90° (SSC signal). Fluorescence light collected at 90° is split off by a series of dichroic mirrors and filters in three different colors and detected by photomultiplier tubes (FL1, FL2, and FL3).

15 mW argon-ion laser light source which provides monochromatic light at 488 nm. The laser beam strikes the cells or other particles which flow in a laminar non-turbulent fluid stream through the measurement region (flow cell). Pure laminar flow is achieved by forcing the jet of fluid containing the sample particles into a sheath fluid flowing under pressure slightly lower than that of the sample fluid (2,3). This enables a sequential flow of primarily single cells, sufficiently separated so that only one cell is subject to measurement. The cells will refract the light from the laser beam and, if present, fluorochromes will be excited and emit fluorescence. The refracted light is measured at two different angles. In forward direction of the laser beam ($<1^{\circ}$-10°), the so-called forward scatter (FSC) or low angle scatter is measured, and at 90° (range ~5°), the so-called sideward scatter (SSC) is measured (Figure 1). The FSC signal is primarily dependent upon volume of the particles or cells, whereas the SSC signal provides a measure of internal organization, cytoplasmic granularity, nuclear density, and external cell structure (1-3). The emitted fluorescence light

TABLE 1. Fluorescent reagents useful for measurements with 488 nm excitation.

Reagents	Examples	Applications
Fluorochromes conjugated to specific antibodies, biotin or streptavidin	- fluorescein isothiocyanate (FITC) - phycoerythrin (PE) - PE-Texas Red tandem - PerCP	- immunological marker analysis - determination of HLA-phenotype - analysis of autoantibodies - blood group serology
Fluorescent DNA and/or RNA binding dyes	- acridine orange (AO) - propidium iodide - ethidium bromide - thiazole orange	- measurement of DNA-content - enumeration of reticulocytes - analysis of NK cell function
Fluorescent Ca^{2+} indicator	- fluo-3	- analysis of lymphocyte activation
Fluorescent bacteria or opsonized latex particles	- FITC-labeled E-coli or latex particles	- analysis of phagocytosis

is collected by optics in the 90° angle and, dependent on the wavelength of the emitted light, the light is split off by a series of dichroic mirrors into two or three beams (Figure 1). Fluorescence light can be measured at 515-545 nm (FL1), 565-605 nm (FL2), and >630 nm (FL3). Table 1 summarizes some fluorescent reagents which can be used in flow cytometry with 488 nm excitation (4). The refracted light or fluorescence light is measured by light detection devices, such as photodiodes and photomultiplier tubes, which convert light into an electric signal. The magnitude of the electric signal is proportional to the amount of light detected, which, in case of fluorescence light, is related to the number of fluorescent molecules (1-4). The FSC signal is detected by a photodiode whereas all 90° signals, which are relatively weak, are detected by photomultiplier tubes (Figure 1). Because the sensitivity of each light detection device depends on the voltage applied and/or amplifier gain setting, it is clear that the parameters are measured as relative intensity. The measurements can be performed with either linear or logarithmic amplification. In case of fluorescence measurements in immunological marker analysis logarithmic amplification is preferred. For each cell or particle the signals produced by the different light detection devices are processed and combined and subsequently sent as so-called list mode data to the computer. Flow cytometry enables a rapid acquisition of information about a large number of cells (250-2500 cells per sec).

Analysis of flow cytometry results

The amount of information obtained during measurement is large and sophisticated computers and software programmes are required for data processing and analysis (3). Typically, 5000-10,000 cells are counted and from each cell results on four or five different parameters are obtained. The results of one parameter can be presented and analyzed by

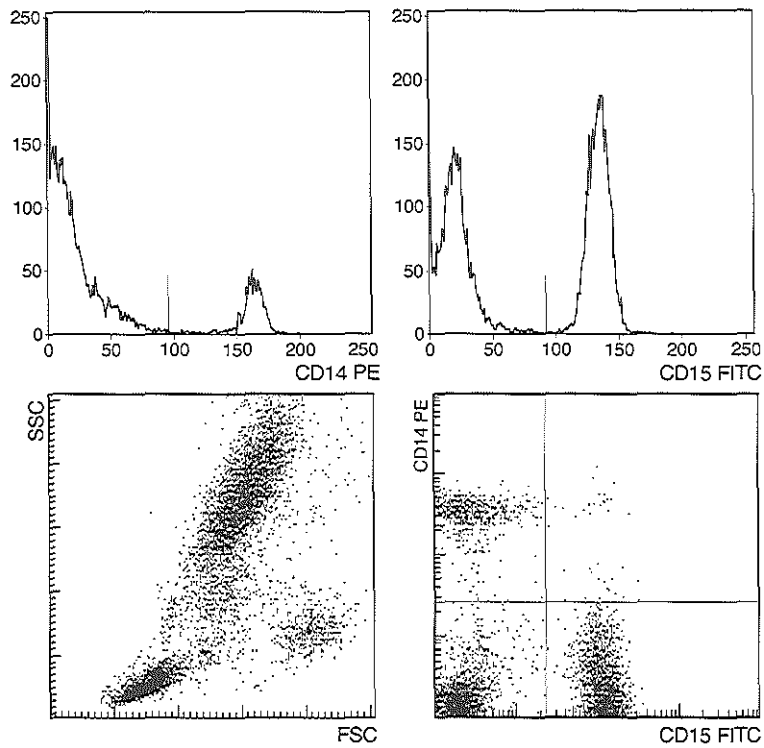

Figure 2. Analysis of CD15 (Leu-M1 FITC), CD14 (My4 PE) double IF staining of lysed whole blood cells obtained from a healthy volunteer. **Upper part:** histogram analysis for CD15 (left) and CD14 (right). **Lower part:** dot plot analysis of FSC against SSC (left) and CD15 against CD14 (right). In the FSC/SSC dot plot debris, lymphocytes, monocytes, and granulocytes can be recognized.

use of histogram analysis (see Figures 2A and 2B). It is also possible to plot two different parameters against each other in one graph, e.g. dot plot diagram, contour plot diagram, or three dimensional profile. Examples of dot plot analysis are given in Figures 2C and 2D. A diagram of FSC against SSC of lysed whole blood cells allows a discrimination between lymphocytes, monocytes, and granulocytes (Figure 2C). Analysis can be performed on all cells or a specific subpopulation can be selected for analysis. In the latter case the cells of interest are selected by "gating" on a specific subpopulation in a histogram or dot plot. A widely used application of the so-called "gating" in immunological marker analysis is the selection of a specific "morphological" cell type in the FSC/SSC dot plot (e.g. blast cells, lymphocytes, or monocytes). The "gated" cells can subsequently be studied for various parameters. An example of "gating" of a specific subpopulation on the expression of a immunological marker, i.e. $CD15^+$ cells in peripheral blood (PB), is given in Figure 3. It is clear that modern flow cytometry enables multiparameter analysis of various cell populations (5).

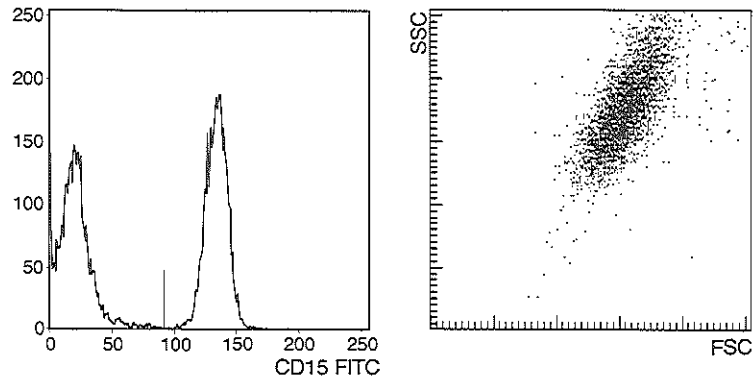

Figure 3. Example of "gating" of a specific subpopulation, i.e. $CD15^+$ cells in lysed whole blood cells obtained from a healthy volunteer. **Left:** histogram analysis of CD15 (Leu-M1 FITC) stained cells. The cells at the right side of the plotted marker are $CD15^+$. **Right:** FSC/SSC dot plot of the "gated" $CD15^+$ cells only. If compared with Figure 2 (lower left) it is clear that $CD15^+$ cells represent the cell population with a variable but high SSC signal.

Diagnostic applications of flow cytometry

Flow cytometry can be used for a wide variety of purposes. In fact, flow cytometry can be performed if the particles of interest are in suspension, if these particles have a diameter which is smaller than 100 μm, and if fluorescence probes are available which are suitable for the specific application (see also Table 1). An extensive discussion of the diagnostic applications of flow cytometry is beyond the scope of this thesis. Table 2 summarizes several applications which are currently used in clinical diagnosis. In this chapter the applications of immunological marker analysis in myeloid disorders, in particular acute myeloid leukemia (AML), will be discussed (Table 3). It should be emphasized that these applications are not restricted to flow cytometry and that other techniques, such as IF microscopy, can be used also.

TABLE 2. Applications of flow cytometry in clinical diagnosis.

Immunological marker analysis of leukocytes	Determination of *in vitro* functions of leukocytes: - analysis of phagocytosis - analysis of NK cell functions - analysis of leukocyte activation *in vitro*
Determination of HLA-phenotype (e.g. HLA-B27)	Blood group serology
Enumeration of reticulocytes	Analysis of malaria parasites in erythrocytes
Measurement of cellular DNA-content: - determination of DNA-ploidy - cell cycle analysis	Determination of total volume of circulating erythrocytes
Analysis of autoantibodies against leukocytes, platelets, or erythrocytes	

TABLE 3. Application of immunological marker analysis in myeloid disorders.

Immunological marker analysis of myeloid malignancies
- immunophenotyping of AML, CML, and true histiocytic NHL
- characterization of subpopulations within one malignancy
- detection of two malignancies in one patient
- recognition of association between immunological phenotypes of AML and specific chromosomal aberrations
- detection of low numbers of AML cells
Analysis of myeloid cells in immunodeficiency
- detection of CD11/CD18 deficiencies in leukocyte adherence deficiency
Characterization of deficiencies of specific antigens
- determination of CD41 deficiency in Glanzmann's thrombasthenia
- determination of CD42 deficiency in the Bernard-Soulier syndrome
- analysis of PI-linked antigens in PNH and AA
Analysis of disorders of maturation of myeloid cells
- characterization of subpopulations in MDS
- analysis of erythroid differentiation in red cell disorders
- analysis of megakaryocytic differentiation in platelet disorders
Analysis of platelet activation

Abbreviation used: CML = chronic myeloid leukemia. For other abbreviations see text.

IMMUNOLOGICAL MARKERS DURING MYELOID DIFFERENTIATION

The expression of immunological markers during normal myeloid differentiation has been summarized in Chapter 2.1. The presence of a continuum of maturational stages of myeloid precursor cells in normal bone marrow (BM) complicates the recognition of distinct cell populations. This might explain the relatively low number of studies on the immunophenotype of myeloid cells and their precursors of normal BM samples (6-12). Using multiparameter flow cytometry Terstappen and Loken could identify consistent patterns of gradual changes of light scattering properties and cell surface antigen expression during normal myeloid differentiation (12). Such consistent characteristics of differentiation pathways in normal individuals might provide a basis for the discrimination between normal and abnormal myeloid differentiation.

In Table 4 we have summarized a few characteristics of the antigens which can be studied and/or used in the diagnosis of myeloid disorders (13-96). For most markers the chromosome localization of the encoding genes has been determined. Human rodent hybrids segregating human chromosomes have been shown useful for gene localization studies (19,47), because they allow to determine the concordancy between the presence and absence of the specific marker and human chromosomes. Either a specific probe, which hybridize to the encoding genes, or monoclonal antibodies (McAb), which bind to the expressed antigens, are useful to determine whether hybrid cells with (well-defined human chromosome content) contain the specific genes (13,19). Chromosomal *in situ* hybridization can be performed to confirm the assignment and to localize the gene to a specific part or

band of the chromosome (31,49). Alternatively, mapping of antigens have been performed by hybridization of a specific probe directly to DNA of human chromosomes which have been sorted by use of flow cytometry (30,35). An example of mapping of a myeloid antigen, i.e. CD33, by use of interspecies hybrid cells and McAb is given in Chapter 2.3.

IMMUNOLOGICAL MARKER ANALYSIS IN AML

Marker expression in AML subtypes

As have been mentioned in Chapter 2.1, myeloid markers in AML are not as informative as the lymphoid markers in acute lymphoblastic leukemia (ALL). Most studies of immunological marker analysis in AML indicate considerable heterogeneity of marker expression between different patients as well as within a given individual (97-102,103). Although a few proposals have been made for an objective immunological classification system of AML, such classifications are not widely used (97,100,101,104). Because both the morphological French American British (FAB) classification and the expression of immunological markers reflect cell lineage and maturation, many investigators have attempted to correlate morphology and immunophenotype (98,99,105-107). In Figure 4 we have tried to indicate the expression of relevant immunological markers for each myeloid differentiation stage as well as the location of the various types of myeloid malignancies. In general, the degree of correlation between immunophenotype and FAB classification is not convincing (98,99,105-107). Nevertheless, associations between FAB type and immunophenotype have been observed. For example, the expression of monocytic antigen CD14 is highly correlated with AML-M4 or M5 (97,98,100,107-112). However, CD14 expression is not completely specific for the monocytic variants of AML and other types of AML may be $CD14^+$ (98,99,107,109,111). Furthermore, CD14 expression is often variable in AML classified as FAB-M5a (98,109,110). For $CD14^-$ AML-M4 and AML-M5 cases CD11c and/or CD36 may be informative to confirm monocytic differentiation (113). One striking correlation is that of FAB-M3 with HLA-DR negativity (97,98,107,109,112,114-116). Interestingly, the less differentiated hypogranular variant of AML-M3 is frequently $HLA\text{-}DR^+$ (114). In addition to expression of myeloid markers CD13 and CD33 AML-M3 is positive for CD9 (115,116). Despite evidence for granulocytic differentiation AML-M3 is frequently negative for CD15 (117,118).

CD34 is a marker of myeloid and lymphoid precursor cells (119-121). Expression of CD34 has been observed in all AML types except AML-M3 (97,111,122-124). Especially, AML-M1 and M2 are usually strongly positive for CD34, indicating the prevalence of a large amount of immature cells. In the other subtypes of AML the proportion of $CD34^+$ cells is often variable and low percentages (<20%) of $CD34^+$ cells can be found in the majority of "$CD34^-$ AML" (124). These observations illustrate that interpretation of immunological marker analysis in heterogeneous disorders, such as AML, often depends on the percentage which is taken as threshold to differentiate between marker postivity or negativity. In nor-

TABLE 4. Characteristics of immunological markers which can be used in the diagnosis of myeloid disorders.

CD[a,b] code	Antibodies[b]	Antigen recognized	Molecular Mass (kDa)	Gene localization	References[c]
1. MYELOID MARKERS					
Pan-myeloid markers					
CD13	My7[d], MCS2[e]	pan-myeloid antigen, aminopeptidase N	gp150	15q25-q26	13-15
CD33	My9[d], Leu-M9[f]	pan-myeloid antigen	gp67	19q13.3	16-18
CDw65	VIM-2[g,h]	fucoganglioside; ceramidedodecasaccharide 40	glycolipid	11q12-qter	19
-	MPO-7[i]	myeloperoxidase (MPO)	gp60/12	17q12-q24	20-22
Monocyte macrophage markers					
CD14	My4[d], Leu-M3[f]	monocytic antigen; PI-linked protein	gp55	5q23-q31	23
CD64	32.2[k]	high affinity Fc receptor for IgG (FcγRI)	gp75	1q21-p22	24,25
CD68	Ki-M6[g], Ki-M7[g]	macrophage antigen	gp110		
-	Monocyte-2/61D3[l]	monocytic antigen	gp75		
-	RFD9[m]	macrophage antigen	gp25		
Myeloid granulocytic markers					
CD15	VIM-D5[g,h], Leu-M1[f]	X-hapten; fucosyl-N-acetyl-lactosamine (FAL)	variable	11q12-qter	19,26
CD66	CLB-gran/10[n]	granulocytic antigen (phosphoprotein)	gp180-200		27
CD67	B13.9[n]	granulocytic antigen; PI-linked protein	p100		
Erythroid markers					
-	CLB-eryH[n]	H antigen; backbone of ABO proteins	variable	9q34.1-q34.2	28
-	VIE-G4[g,h], CLB-ery/1[n]	glycophorin A (GpA)	gp41	4q28-q31	29
Megakaryocytic platelets markers					
CD41	J15[l], CLB-thrombo/7[n]	GPIIb-GPIIIa complex (Glanzmann antigen)	gp145/115	17q21.32[o]	30-32
CD42a	FMC25[d]	platelet GPIX] receptor for von	gp20	3	33
CD42b	AN51[f]	platelet GPIb] Willebrand factor	gp170	17p12-pter	34
CD61	CLB-thrombo/1[n]	integrin β3 chain (GPIIIa; VNR-β chain)	gp115	17q21.32[o]	31,35,36
2. LYMPHOID MARKERS WITH CROSS LINEAGE EXPRESSION ON MYELOID CELLS					
CD1	Leu-6[f], OKT6[p]	common thymocyte antigen (T6); MHC like protein	gp43/45/49	1q22-q23	37,38
CD2	T11[d], OKT11[p]	T11 antigen; SRBC receptor; LFA-2	gp50	1p13	39-44
CD4	Leu-3a[f], OKT4[p]	T4 antigen; HIV receptor	gp60	12pter-p12	45-47
CD7	Leu-9[f], 3A1[q]	Tp41 antigen; Fc receptor for IgM (FcμR)	gp41	17q25.2-q25.3	47-51
CD10	VIL-A1[g,h], J5[d]	CALLA; neutral endopeptidase	gp100	3q21-q27	52,53
CD19	B4[d], Leu-12[f]	pan-B cell antigen	gp90		54
CD56	Leu-19 (My31)[f]	NCAM; PI-linked and transmembrane-forms	gp135-220	11q23	55,56

CD[a,b] code	Antibodies[b]	Antigen recognized	Molecular Mass (kDa)	Gene localization	References[c]
3. MARKERS WHICH ARE NOT RESTRICTED TO ONE DIFFERENTIATION LINEAGE					
CD9	BA-2[f]	p24 antigen; role in platelet aggregation	p24	12p13	57-60
CD11a	CLB-LFA-1/2[n]	LFA-1 antigen (αL chain)	gp180	16p13.1-p11	61,62
CD11b	Mo1[d], OKM1[p]	MAC-1 antigen (αM chain); C3bi receptor (CR3)	gp155	16p13.1-p11	62,63
CD11c	Leu-M5/SHCL3[f]	p150.95 antigen (αX chain);	gp150	16p13.1-p11	62
CD16	Leu-11b[f]	FcγRIII; PI-linked protein	gp50-65	1q23	64-66
CD18	CLB/LFA-1/1[n]	integrin β2 chain	gp95	21q22.3	61,67,68
CD23	Leu-20[f]	low affinity Fc receptor for IgE (FcεRII)	gp45	19q13.1-pter	69
CD24	BA-1[r]	granulocytic (PI-linked) and B cell antigen	gp42		70,71
CDw32	IV.3[k]	intermediate affinity Fc receptor for IgG (FcγRII)	gp40	1q23-q24	65,72-74
CD34	HPCA-2[f], BI-3C5[s]	precursor antigen	gp110	1q32	75-79
CD36	OKM5[p], ESIVC7[n]	thrombospondin receptor (platelet GPIV)	gp90		80
CD38	Leu-17[f], OKT10[p]	T10 antigen	gp45	4	57
CD54	Leu-54[f], BBL-4[t]	intracellular adhesion molecule-1 (ICAM-1)	gp90	19p13.3-p13.2	81
CD55	BRIC110[u]	decay accelerating factor (DAF); PI-linked	gp70	1q32	82-85
CD58	G26[g], TS2/9[v]	LFA-3; PI-linked and transmembrane forms	gp40-65	1p13	43,86-88
CD59	YTH53.1[w]	Ly-6 antigen; PI-linked protein	gp18-20	11p14-p13	89,90
CD71	T9[d], OKT9[p]	T9 antigen; transferrin receptor	gp190	3q26.2-qter	91-93
-	L243[f], OKIa[p]	HLA-DR, non-polymorphic antigen	gp29/34	6p21.3	94
-	anti-TdT[x]	terminal deoxynucleotidyl transferase (TdT)	p58	10q23-q24	95,96

a. CD = cluster of differentiation, as defined during the Leukocyte Typing Conferences (Paris, France, 1982; Boston, MA, USA, 1984; Oxford, UK, 1986; Vienna, Austria, 1989).
b. 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. For references on the monoclonal antibodies see Table 1 of Chapter 2.1. In this Table only references on additional characterization of the antigens and especially on the chromosome localization of the encoding genes are given.
d. Coulter Clone, Hialeah, FL, USA.
e. Nichirei Co., Tokyo, Japan.
f. Becton Dickinson, San Jose, CA, USA.
g. Behring, Marburg, Germany.
h. Dr. W. Knapp and Dr. O. Majdic, Vienna, Austria.
i. DAKOPATTS, Glostrup, Denmark.
k. Medarex inc., West Lebanon, NH, USA.
l. Bethesda Research Laboratories, Gaithersburg, MD, USA.
m. Royal Free Hospital, London, UK.
n. Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands; in other countries: Janssen Biochimica, Beerse, Belgium.
o. Both GPIIb and GPIIIa genes have been mapped on chromosome 17q21.32.
p. Ortho Diagnostic Systems, Raritan, NJ, USA.
q. American Type Culture Collection, Rockville, MD, USA.
r. Hybritech, San Diego, CA, USA.
s. SeraLab, Crawley Down, UK.
t. British Biotechnology, Oxford, UK.
u. Dr. D. Anstee, Bristol, UK.
v. Dr. T. Springer, Boston, MA, USA.
w. Dr. H. Waldmann, Cambridge, UK.
x. Supertechs, Bethesda, MD, USA.

mal BM <1%-4% $CD34^+$ cells can be found indicating that a cut off value of 10% or even lower may be justified (6,119-121). In two AML-M3 cases tested we could identify 6% and 17% of $CD34^+$ cells (see also Chapter 4.2).

For the diagnosis of AML-M6 markers, such as CD36, H antigen, and glycophorin A have proved to be useful (101,125-127). However, both CD36 and H antigen are not erythroid lineage specific and glycophorin A is only expressed in a part of AML-M6 patients. More specific for AML-M6 is a $CD36^+$, $HLA\text{-}DR^-$ phenotype (125). Results of immunological marker analysis of AML-M6 cases confirmed the presence of erythroid and myeloid subpopulations, which is in line with multilineage involvement in this type of AML (124,125).

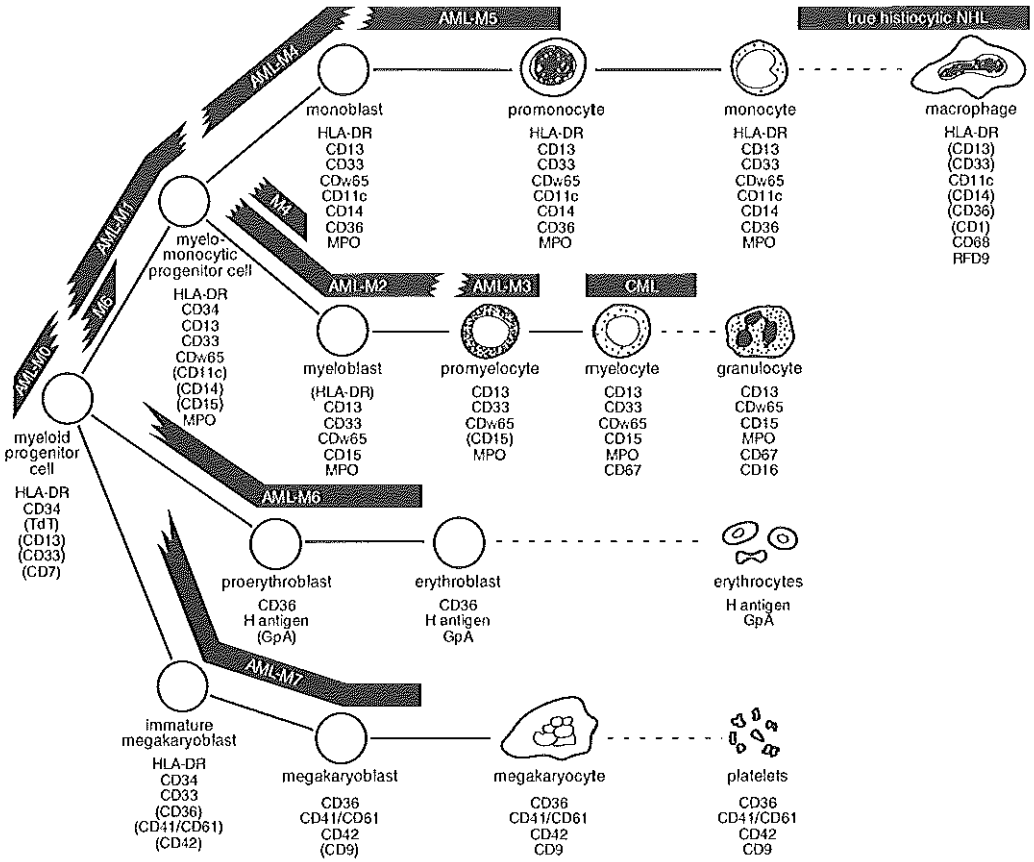

Figure 4. Hypothetical scheme of myeloid differentiation. The expression of relevant immunological markers is indicated for each differentiation stage; markers in parentheses are not always expressed. The bars represent the various types of leukemias and non-Hodgkin lymphomas and indicate where these malignancies can be located according to their maturation-arrest. It should be emphasized that most acute leukemias of the myeloid lineage have a heterogeneous phenotype, i.e. are composed of cells in multiple immature myeloid differentiation stages. To underline this phenotypic heterogeneity, several bars fade into each other. **Abbreviation used:** CML = chronic myeloid leukemia. For other abbreviations see text.

As has been mentioned in Chapter 1 expression of platelet markers (i.e. CD41, CD42a, CD42b, CD61, or antibodies against factor VIII related antigen) has been incorporated in the FAB criteria for the diagnosis AML-M7 (128,129). In the majority of AML-M7 cases published the blast cells expressed several of these platelet markers (130-132).

Due to the fact that expression of some pan-myeloid markers (e.g. CD13 or CD33) is required for the diagnosis AML-M0, it is logical that there is an excellent correlation between AML-M0 and the expression of these myeloid markers (133-136). It has been suggested that, similar to cytoplasmic expression of the lymphoid antigens CD3 and CD22 in precursor T and B cells, respectively, CD13 is expressed in the cytoplasm of immature myeloblast (137). Therefore, cytoplasmic CD13 staining may contribute to the characterization of immature types of acute leukemia (137). Besides expression of CD13 and/or CD33, leukemias classified as AML-M0 should be negative for lymphoid markers (133). The presence of myeloperoxidase (MPO) can be demonstrated by electron microscopy (EM) and/or immunocytochemistry. McAb directed against MPO have proved to be valuable reagents for AML diagnosis (138,139), although it has been claimed that conventional enzym cytochemistry methods are equally sensitive (140). According to a recent study anti-MPO McAb are positive in the majority of AML-M0 patients, indicating that, by analogy with AML-M7 diagnosis, AML-M0 can be diagnosed in most cases by use of immunological markers and EM studies are only necessary in a minority of cases (141). Despite the application of myeloid markers in the diagnosis of immature types of acute leukemia, a small subgroup of cases (<1% of acute leukemia) remain unclassifiable (141-145). According to Goasguen and Bennett, only those patients should be diagnosed as having acute undifferentiated leukemia (AUL) (142). Alternatively, for those cases, which might express CD7, CD34, HLA-DR, and/or TdT, the term "stem cell" leukemia has been proposed (143). It has been suggested that detection of MPO mRNA or induction of MPO by in *in vitro* culture might be useful for the distinction between immature variants of AML and ALL (146,147). Probably due to the low number of patients, the prognosis of leukemias with a stem cell phenotype is not clear.

It is now generally accepted that FAB typing and immunophenotyping provide complementary information and that the best method for typing acute leukemias is the combined use of cytomorphology, cytochemistry, and immunological marker analysis.

Cross-lineage marker expression in AML

Numerous reports of AML cases expressing lymphoid markers have been published. In particular, these concern the B cell marker CD19 and the T cell markers CD2, CD4, and CD7 (Table 4) (103,148-159). On the other hand, cross-lineage expression of myeloid markers in ALL has been described (reviewed in 160). Such observations have been interpreted as lineage infidelity and also terms, like hybrid acute leukemia and mixed lineage leukemia, have been used to describe these acute leukemias (161-164). Furthermore, the

terms biphenotypic acute leukemia and biclonal acute leukemia are used, referring to co-expression of lymphoid and myeloid markers on one cell population or to the expression of these two markers on different cell populations, respectively (162). It has been proposed that cases of mixed lineage acute leukemia reflect genetic misprogramming because of aberrant gene expression in these leukemias (161,164). However, according to Greaves et al., these merely reflect the existence of normal immature cells expressing lymphoid and myeloid genes (165). The findings of CD4 and CD7 expression on immature cell types in normal BM and the expression of CD4 on subpopulations of monocytes are in support for the hypothesis of Greaves et al. (166-168). Expression of CD7 especially occurs in immature types of AML, whereas CD2 and CD4 expression have been demonstrated in both immature and monocytic types of AML (103,150,154,156,169-172). Multidimensional flow cytometric analysis could demonstrate that in most AML patients with cross-lineage marker expression non-myeloid antigens were homogeneously expressed by the leukemic cells (103). In Chapter 3.3 we present evidence that AML-M4 with BM eosinophilia (M4Eo) exhibits a specific $CD2^+$ immunophenotype.

Clinical significance of the expression of specific markers in AML

The expression of certain immunological markers on AML cells may have prognostic significance. The myeloid markers CD13 and CD14 have been found to predict for a low rate of complete remission (CR) upon standard chemotherapy (109,112,173-177). Other reports demonstrated lower CR rates and a worse prognosis for patients having a $CD34^+$ AML (i.e. >10% or >20% of $CD34^+$ cells) (111,122-124,176-179). However, these AML patients more frequently displayed chromosomal abnormalities involving chromosomes 5 and/or 7, which are also known as factors with a bad prognosis (122,123). The reported lower CR rates for H $antigen^+$ or platelet $marker^+$ AML corresponds with the observations that AML-M6 and AML-M7 have a worse prognosis (173,174). Expression of CD33 in AML was found to be of no clinical relevance (112). Nevertheless, results of recent observations suggest that therapy outcome might be related to the intensity of CD33 expression of the leukemic cells (175,179). In five studies expression of CD15 in AML was associated with longer survival (111,112,174,176,180).

In general, expression of CD7 in AML is associated with a poor prognosis (169,171). Reports on the prognostic value of the expression of other lymphoid markers in AML are contradictious (149,177,181). In a recently published study on the expression of CD2, CD4, and CD19 in childhood AML no prognostic significance was found (172). Interestingly, some distinct biological features, such as lymphadenopathy and skin infiltration, occur in higher frequencies in T cell $marker^+$ AML (152,156).

With regard to the prognostic significance of immunological marker analysis in AML, it can be concluded that immature phenotypes ($CD7^+$, $CD34^+$), monocytic phenotypes ($CD14^+$), and erythroid and megakaryocytic phenotypes may be related to a worse

prognosis, whereas granulocytic differentiation ($CD15^+$) of AML is probably related to a better prognosis. However, studies of larger populations will be needed to determine the precise prognostic value of specific immunophenotypes.

Characterization of subpopulations in AML

Immunological marker analysis is useful to determine the immunophenotype of the various subpopulations which are present in heterogeneous AML. Especially double IF staining can be helpful to discriminate various immature and more mature subpopulations (see Chapters 2.1 and 3.3 for examples). In addition, morphological parameters, such as phase contrast microscopy or FSC and SSC, can be used to recognize different subpopulations. Figure 5 demonstrates the value of using SSC to discriminate between immature, partly $CD34^+$ cells and more mature $CD15^+$ cells in the BM of an AML patient. In Chapter

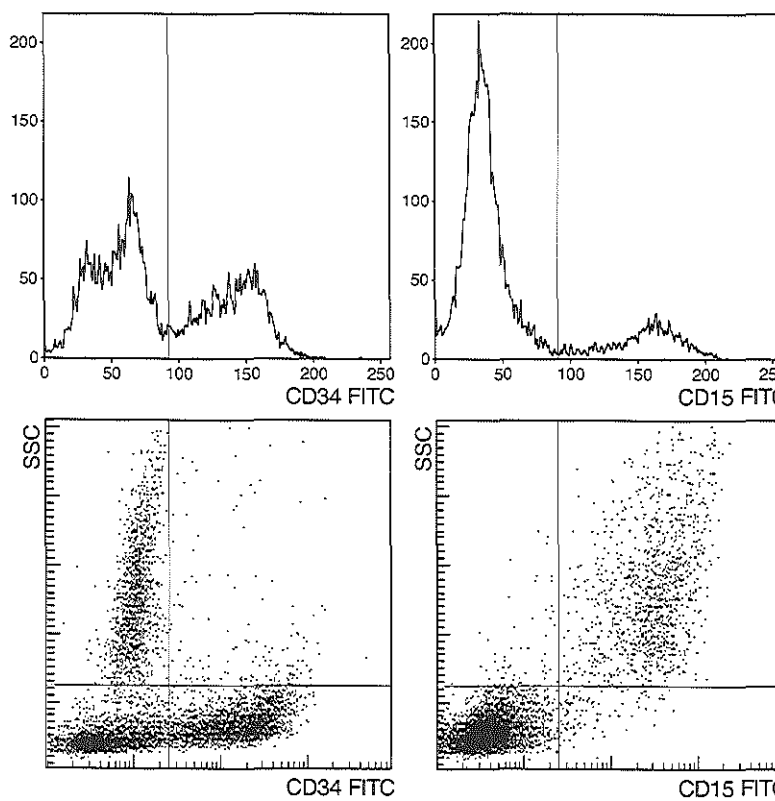

Figure 5. Comparison of one parameter (histogram) analysis and two parameter (dot plot) analysis of CD15 and CD34 labelings of an AML-M2 (patient Y.C.;37,F) at diagnosis. **Left:** analysis of CD34 stained cells. **Right:** analysis of CD15 stained cells. Both markers are analyzed by use of histogram analysis (above) and dot plot analysis against SSC (below). It is clear that dot plot analysis gives additional information. Cells with a low SSC signal (e.g. blast cells) are partly $CD34^+$ whereas the $CD15^+$ cells exhibit a high SSC signal.

4 the relevance of the detection of AML subpopulations which express the nuclear enzyme terminal deoxynucleotidyl transferase (TdT) will be discussed extensively.

Finally, in AML, immunological marker analysis can be used for the detection of low numbers of leukemic cells (183). In Chapter 4 we will demonstrate that double IF staining for TdT and myeloid markers using fluorescence microscopy represents a powerful tool for the detection of residual AML cells. For the detection of low numbers of AML cells by use of flow cytometry double and triple IF stainings have been proposed for combinations of surface membrane markers which are expressed on AML blasts but which seems to be absent or extremely rare in normal BM (184,185). In experiments using mixtures of AML cells and normal BM, it was claimed that 0.01%-1% of AML cells could be detected (184,185). However, data from follow-up of AML patients using these types of double and triple IF stainings in flow cytometry are still lacking. In our opinion it is difficult to use flow cytometry for proper analysis of low percentages of cells (<0,1%-1%) in heterogeneous cell populations such as BM or PB, especially in regenerating BM after termination of cytostatic therapy. Furthermore, since immunological markers are not leukemia specific, the application of specific marker combinations for the detection of residual disease requires experiments on the occurrence of these markers in normal samples, at different ages as well as samples from patients during and after cytostatic treatment.

IMMUNOLOGICAL MARKER ANALYSIS IN NON-MALIGNANT MYELOID DISORDERS

The possibilities to use immunological marker analysis in non-malignant myeloid disorders are summarized in Table 3. Leukocyte adhesion deficiency is a rare autosomal recessive disorder with impaired chemotaxis and phagocytosis. This is caused by a deficient surface membrane expression of CD11/CD18 molecules due to defects in the common CD18 subunit of these heterodimers (186). In patients with Glanzmann's thrombasthenia the expression of the CD41 antigen by platelets is reduced or absent, whereas in patients with the Bernard-Soulier syndrome the expression of CD42 is deficient (187-189). Immunological marker analysis is useful in the analysis of these two platelet markers. Flow cytometric analysis also allow the identification of heterozygote carriers of Glanzmann's disorder (189).

Paroxysmal nocturnal hemoglobinuria (PNH) is an acquired clonal disorder characterized by recurrent hemolysis, resulting in anemia, hemoglobinuria, thrombosis, infections, and a tendency toward BM aplasia (190). The common defect is a deficiency of glycosyl phosphatidylinositol (PI) anchored surface membrane molecules (190-193). These include CD14 on monocytes, CD16, CD24, and CD67 on granulocytes, and CD48, CD55, and CD59 on erythrocytes (Table 4). Interestingly, deficiency of PI linked antigens have recently been demonstrated in a few patiens having aplastic anemia (AA) (194). Flow cytometry, using double IF staining is a powerful tool to determine the expression of PI linked molecules on various cell types (191-193). An example of such analyses, performed on PB

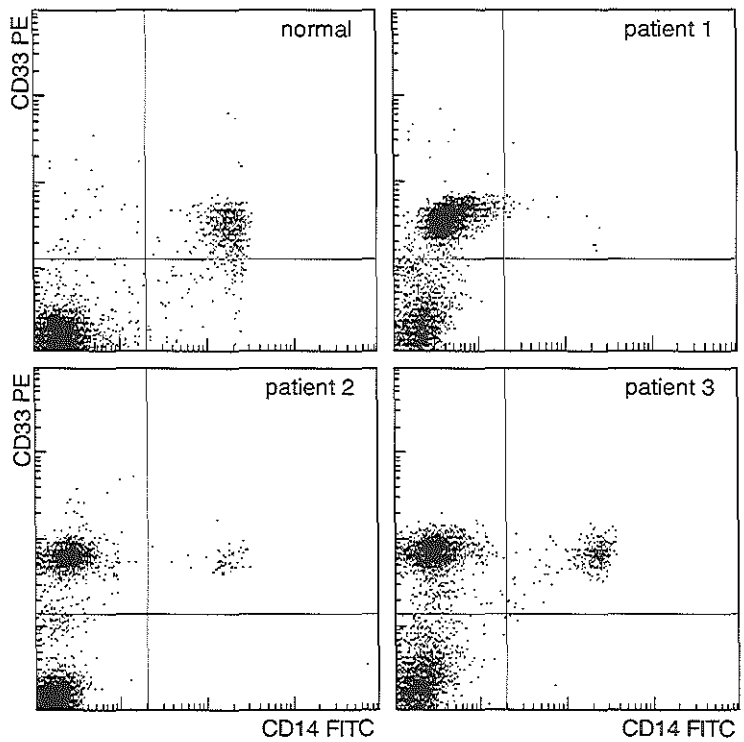

Figure 6. Dot plot analysis of CD14 (My4 FITC), CD33 (My9 PE) double IF staining of PB MNC cells from one healthy volunteer and three patients with PNH. The analyses are performed within a gated cell population including lymphocytes and monocytes, but excluding debris and granulocytes. In the healthy volunteer virtually all $CD33^+$ cells are $CD14^+$. In the three PNH patients virtually all $CD33^+$ cells (patient 1) or the majority of the $CD33^+$ cells lack the PI-linked CD14 antigen, indicating monocyte subpopulations with CD14 deficiency.

cells from a PNH patient, is given in Figure 6.

Reports on the use of immunological marker analysis in the diagnosis of myelodysplastic syndromes (MDS) are scarce (195). It proved to be useful to detect immature $CD34^+$ cells in MDS types with an excess of blast cells. High expression of CD34 was found to be related to MDS with bad prognosis. Moreover, immunophenotyping have been used in advanced microscopic methods to perform karyotypic analysis or DNA *in situ* hybridization in immunologically defined cell types (196). Flow cytometry might be useful in the analysis of disorders of megakaryopoiesis or erythropoiesis, but reports on these applications are scarce (7,197). Finally, due to the fact that flow cytometry enables quantification of fluorescence signals, some platelet functions can be analyzed. It has been found that activation of platelets will result in an upregulation of the expression of various platelet markers (198).

TABLE 5. Comparison of flow cytometry and IF microscopy for immunological marker analysis.

Flow cytometry	IF microscopy
Favorable	**Favorable**
- rapid analysis of large numbers of cells - quantification of fluorescence intensity - multiparameter analysis of cell populations - automatic data handling (calculation, printed report of results, data storage)	- integrated information of (phase contrast) morphology and fluorescence staining patterns per cell - simple analysis of intracellular markers (cytoplasmic and/or nuclear) - detection of low frequencies of cells (down to 0.01% or even 0.001%) - analysis of cell samples with low cell count (<1000 cells) by use of cytocentrifuge preparations
Unfavorable	**Unfavorable**
- "morphological" (scatter) information is restricted to cell populations (analysis at the single cell level is not possible) - no information about expression patterns at the single cell level (this particularly concerns staining patterns of intracellular antigens) - restricted possibilities to determine the precise proportion of rare events - proper analysis of cell samples with low cell count (<1000 cells) is not possible	- laborious - analysis of large numbers of cells is only possible by screening for positivity - quantification of fluorescence intensity is not possible - no automatic data handling - experience in recognition of specific fluorescence staining patterns is a prerequisite
Costs (per flow cytometer)	**Costs (per fluorescence microscope)**
- initial expenses 90,000 ECU - expenses for maintenance 6,000 ECU - total costs per year 24,000 ECU	- initial expenses 16,000 ECU - expenses for maintenance 2,000 ECU - total costs per year 3,000 ECU

LIMITATIONS OF FLOW CYTOMETRY

The various possibilities of flow cytometry have been outlined above and are summarized in Table 5. In this table the possibilities and limitations of flow cytometry and IF microscopy are compared. Flow cytometry enables a rapid acquisition of a large number of cells, quantification of fluorescence signals, and automatic data processing and storage. Multiparameter analysis can be performed, which has proved to be useful in analysis of heterogeneous cell populations. Although it has been claimed that flow cytometry is an objective method for cell analysis, it should be remarked that "gate setting" and "marker setting" is often performed manually. This needs experience and knowledge concerning the characteristics of normal and abnormal cell populations. Especially in case of heterogeneous cell populations gate setting is difficult and relevant subpopulations may be missed. Therefore, we recommend to analyse in a "total cell" gate first (only excluding debris), before analysis of specific "gated" subpopulations is performed. A limitation of flow cytometry is that the analyses are restricted to cells in suspension. Furthermore, the analysis of intracellular antigens is difficult (Table 5). Some recently published protocols for

intracellular staining have proved to be useful (199-201). However, high background staining and cell destruction hamper the precise determination of positivity. Moreover, it has to be tested whether the various normal and malignant cell types are equally sensitive for these fixation protocols.

The major advantage of IF microscopy is the possibility to analyze fluorescence staining and phase contrast at the single cell level, which is particularly important for detection of low frequencies of tumor cells (Table 5). If flow cytometry is used for immunophenotyping of hematopoietic malignancies, a fluorescent microscope is highly recommended for specific applications, such as intracellular staining, detection of rare events, and the recognition of specific fluorescence patterns.

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CHAPTER 2.3

EXPRESSION OF THE MYELOID DIFFERENTIATION ANTIGEN CD33 DEPENDS ON THE PRESENCE OF HUMAN CHROMOSOME 19 IN HUMAN-MOUSE HYBRIDS*

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SUMMARY

Interlineage human-mouse hybrids were constructed by fusion of human acute myeloid leukemia (AML) cells, classified as AML-M0, with the mouse thymoma cell line BW5147. Some of the hybrids expressed the human differentiation antigens CD4, CD7, CD33, and CD71 (transferrin receptor). Chromosome analysis revealed that the expression of the myeloid antigen CD33 is dependent on the presence of human chromosome 19, which is in agreement with the location of CD33 coding sequences on chromosome 19, as recently reported by Peiper et al. (1). Furthermore, these hybrids allowed us to confirm the assignment of the CD4 antigen, the CD7 antigen, and the CD71 antigen to human chromosomes 12, 17, and 3, respectively.

INTRODUCTION

The myeloid antigens CD13(gp150) and CD33(gp67), which can be recognized by the monoclonal antibodies (McAb) My7 and My9, respectively, are expressed by human monocytes, promyelocytes, and myeloid blasts (2). Also some of the acute undifferentiated leukemias and acute lymphoblastic leukemias (ALL) appear to express these myeloid antigens (3,4). Using a tertiary mouse cell transformant containing amplified gp150 coding sequences, Look et al. (5) cloned the CD13 coding gene and assigned this gene to human chromosome 15. The CD33 gene was isolated by Peiper et al. (6) who could assign this gene to chromosome 19 in a Southern blot experiment. Recently, they sublocalized the CD33 gene to

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the q13.3 region of chromosome 19 by in situ hybridization on metaphase chromosomes from peripheral blood (PB) lymphocytes (6). Whether expression of the CD33 antigen depends on the presence of chromosome 19 could not be tested in these experiments. We have constructed human-mouse hybrids to study the expression of the CD33 antigen.

MATERIALS AND METHODS

Cells and fusion

Human leukemic cells were obtained from patient S.E., a 19-year-old man suffering from an AML-M0. Mononuclear cells (MNC) were isolated from PB by Ficoll Paque (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) centrifugation.

As rodent partner for fusion we used the hypoxanthine phosphoribosyl transferase deficient (HPRT$^-$) AKR thymoma cell line, BW5147. Cell fusion was performed as described (7) using polyethylene glycol as fusogen and a medium containing hypoxanthine (10^{-4} Mol), aminopterin (10^{-7} Mol), and thymidine (10^{-5} Mol) to select hybrid cells. The hybrid clones were grown in suspension RPMI 1640 culture medium supplemented with 15% foetal calf serum, glutamine, and antibiotics. One hybrid cell line was subcloned by limiting dilution in microtitre plates.

The same populations of cells were used for karyotyping and immunological marker analysis.

Immunological marker analysis of the AML cells and the hybrid clones

PB MNC from patient S.E. as well as 32 hybrids were incubated with optimally titrated relevant McAb as described (8). Detailed information about the McAb used is summarized in Table 1. As a second step reagent we used a fluorescein isothiocyanate conjugated goat anti-mouse immunoglobulin antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). The AML cells were also tested for the expression of terminal deoxynucleotidyl transferase (TdT) as described (8). The immunofluorescence labelings were analyzed on Zeiss microscopes (Carl Zeiss, Oberkochen, Germany), equipped with phase contrast facilities. A hybrid clone was scored as positive for an immunological marker if more than 10% of the cells expressed this marker.

Chromosome analysis

Air dried chromosome spreads were R-banded with acridine orange after heat denaturation. At least 16 metaphases of each hybrid cell line were analyzed. A hybrid clone was scored positive if more than two metaphases out of 16 contained the particular human chromosome.

RESULTS

The results of the immunological marker analysis of the AML-M0 cells are given in Table 1. The cells were positive for CD4, CD7, CD33, CD71, and HLA-DR; the expression of both CD4 and CD33 was weak. About 7% of the AML cells expressed TdT.

Fusion of the AML cells with the AKR thymoma cell line BW5147 resulted in 140 primary and 18 secondary clones. Thirty-two of these hybrid clones were studied by both immunologi-

TABLE 1. Immunological marker analysis of the human AML-M0 cells.

CD[a] (antibodies)	Antigen recognized	Positive PB cells (%)
CD2 (T11)[b]	T11 antigen	1
CD3 (Leu-4)[c]	T3 antigen	1
CD4 (Leu-3)[c]	T4 antigen	34[h]
CD5 (Leu-1)[c]	T1 antigen	2
CD7 (3A1)[d]	Tp41 antigen	55
CD8 (Leu-2)[c]	T8 antigen	0.5
CD13 (My7)[b]	pan-myeloid antigen	0
CD14 (My4)[b]	monocytic antigen	0
CD15 (VIM-D5)[e]	myeloid antigen	1
CD19 (B4)[b]	B cell antigen	3
CD33 (My9)[b]	pan-myeloid antigen	60[h]
CD71 (661G10)[f]	transferrin receptor (T9 antigen)	94
anti-TdT[g]	terminal deoxynucleotidyl transferase	7
anti HLA-DR[c]	HLA-DR, non-polymorphic antigen	81

a. Cluster of differentiation as proposed by the Workshops on Human Leukocyte Differentiation Antigens (Paris, France, 1982; Boston, MA, USA, 1984; Oxford, UK, 1986; Vienna, Austria, 1989).
b. Coulter Clone, Hialeah, FL, USA.
c. Becton Dickinson, San Jose, CA, USA.
d. American Type Culture Collection, Rockville, MD, USA.
e. Dr. W. Knapp, Vienna, Austria.
f. Dr. J.M. van de Rijn, Amsterdam, The Netherlands.
g. Supertechs, Bethesda, MD, USA.
h. Weak antigen expression.

cal marker analysis and cytogenetics. Together these 32 hybrids contained all human chromosomes except for chromosome 9.

Fourteen clones out of the 32 hybrids tested expressed the CD33 antigen. We found a high concordancy rate (97%) between the absence and the presence of the CD33 antigen and chromosome 19 (Table 2). Fifteen clones expressed the CD7 antigen and 24 clones expressed the CD71 antigen. We also found a high concordancy (97%) between the presence and absence of the CD7 antigen and chromosome 17 and a complete concordancy (100%) between the presence and absence of the CD71 antigen and chromosome 3 (Table 3). CD4 was expressed by 20 clones which all contained human chromosome 12. On the other hand, only 77% of the chromosome 12 containing hybrids were $CD4^+$ (Table 3). In three out of the six chromosome 12^+, $CD4^-$ hybrids <1% $CD4^+$ cells were seen.

DISCUSSION

The CD33 antigen was expressed by 15 of the 32 hybrids. Cytogenetic analysis of these hybrids allowed us to confirm the assignment of the CD33 encoding genes to human chromosome 19 (1,6). The concordancy rate between the absence and presence of CD33 expression and chromosome 19 was 97% (Tables 2 and 3). In their mapping experiments Peiper et al. (1) used a panel of somatic cell hybrids, obtained from fusion of human and hamster fibroblasts. These hybrids were tested for the presence of the gp67 coding gene by

TABLE 2. Relationship between the presence of human chromosomes and expression of CD33 in 32 hybrid clones.

Human chromosome	Chromosome/CD33 (no. of clones)				Discordancy (%)
	+/+	+/−	−/+	−/−	
1	11	12	3	6	47
2	11	11	3	7	44
3	12	12	2	6	44
4	6	11	8	7	59
5	7	8	7	10	47
6	11	12	3	6	47
7	9	12	5	6	53
8	8	12	6	6	56
9	0	0	14	18	44
10	10	14	4	4	56
11	9	11	5	7	50
12	11	11	3	7	44
13	6	11	8	7	59
14	11	13	3	5	50
15	9	12	5	6	53
16	10	11	4	7	47
17	9	7	5	11	38
18	1	4	13	14	53
19	14[a]	1[b]	0	17	3
20	7	10	7	8	53
21	9	11	5	7	50
22	10	11	4	7	47
X	13	18	1	0	59
Y	0	0	14	18	44

a. One of these CD33 positive hybrids only contained human chromosomes 19 and X.
b. This hybrid clone contained chromosome 19 in 13 out of 28 examined metaphases while no CD33 positivity was found. This may be due to an undetected chromosomal rearrangement.

use of Southern blotting with a CD33 probe, but it was not reported whether the hybrids expressed CD33 on their cell surface. Probably these fibroblast hybrids do not express human myeloid antigens, because expression of differentiation linked or specialized gene products depends on the differentiation lineage of the parental rodent and human cells (9-12). In this respect, it is remarkable that the pan-myeloid antigen CD33 is expressed by hybrids derived from fusion of AML-M0 cells with a mouse T cell line. However, the finding of CD33 positive ALL suggests that CD33 is not entirely myeloid specific and may be expressed on lymphoid cells as well (3). Using immunoselection of transfected COS cells, another CD33 encoding cDNA was isolated recently by Simmons & Seed (13). However, they did not use this probe to assign the CD33 gene.

Our hybrids also expressed the human transferrin receptor CD71 as well as the T cell antigens CD4 and CD7. We could confirm the assignment of the CD7 antigen to chromosome 17 and the CD71 antigen to chromosome 3 (Table 3) (7,14,15). The T cell antigen CD4, which had already been localized on chromosome 12 (7) was found on 77% of the chromosome 12 containing hybrids (Table 3). However, in three out of the six chromosome 12^+, $CD4^-$ hybrids <1% $CD4^+$ cells were seen. Interestingly, only 35% of the AML cells were positive for this

TABLE 3. Number of hybrid clones that show concordancy and discordancy between the presence and absence of human chromosomes and antigen expression.

Human chromosome/ Human membrane antigen	Chromosome 12/ CD4 antigen		Chromosome 17/ CD7 antigen		Chromosome 19/ CD33 antigen		Chromosome 3/ CD71 antigen	
+/+ +/−	20	6[a]	15	1	14	1	24	0
−/+ −/−	0	6	0	16	0	17	0	8

a. In 3 of the 6 chromosome 12^+, $CD4^-$ hybrids <1% $CD4^+$ cells were seen.

T cell antigen. It may be possible that the variable expression of CD4 by the hybrids is related to variable expression of CD4 by the parental leukemic cells.

Our combined results suggest that expression of human differentiation antigens by human-mouse hybrids is not only influenced by the differentiation lineage or differentiation stage of the rodent fusion partner but also by the differentiation stage and antigenic make-up of the human fusion partner.

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CHAPTER 3

IMMUNOPHENOTYPE OF ACUTE MYELOID LEUKEMIAS WITH SPECIFIC CHROMOSOME ABERRATIONS

CHAPTER 3.1

CHROMOSOME ABNORMALITIES IN AML

INTRODUCTION

Non-random clonal chromosome abnormalities are found in 55% to 85% of patients with *de novo* acute myeloid leukemia (AML) and in 70% to 90% of patients with secondary AML (1-8). Karyotypic abnormalities may be numerical, meaning loss, or gain of entire chromosomes (e.g. -7 or +8), or they may be structural (1,9,10). Common structural changes are translocations (t), deletions (del), and inversions (inv). Table 1 summarizes the most common recurring abnormalities in AML in order of decreasing frequency (1-11).

Since the resolution of cytogenetic techniques is not more than a single chromosome band, breaks which are assigned to the same band do not necessarily occur at the same site in molecular terms (1,9). Therefore, aberrations which involve relocation or loss of upto ~3000 kilobases (kb) of DNA (~100 genes) may occur without being detectable by classical cytogenetics. This implies that the finding of a normal karyotype in about one third of AML patients does not exclude the occurrence of somatic mutations, "small" deletions, or other submicroscopical aberrations in these cases.

During the last decade researchers have focused on DNA regions involved in recurrent chromosome changes in malignancies, such as AML. Several oncogenes and other genes involved in growth and differentiation have been associated with these aberrations, since they map in or near the breakpoint areas at the cytogenetic level of resolution (1,12). However, at the molecular level many of these genes are located at a significant distance from the breakpoints and they were not found to be rearranged or abnormally expressed in AML patients with specific chromosome abnormalities (1,12). Table 2 summarizes the genes which have proven to be involved in specific chromosome aberrations. Cytogenetic variants (i.e. the same type of leukemia with chromosome aberrations in which at least one of the breakpoints is comparable) have been helpful to indicate which of the two standard breakpoints is of critical biological significance (Table 1).

As has been discussed in Chapter 1, the association between particular recurrent chromosome abnormalities and specific AML subtypes has substantiated the importance of karyotypic changes in the biology of AML. Specific abnormalities are not only associated with specific morphological and immunological phenotypes, but also with clinical features, responses to treatment, and survival (Tables 1 and 3) (1-11,13-16). Extensive reviews on chromosome aberrations in AML have been published by others (1,9,10). This chapter summarizes information about specific features associated with AML with t(8;21), t(15;17), 11q23 abnormalities, and -5/del(5q) or -7/del(7q). In particular, data on associations

TABLE 1. Common cytogenetic abnormalities associated with AML[a].

Chromosome abnormality[b]	Frequency[c]	Common features
t(8;21)(q22;q22)	5-8	AML-M2, Auer rods
+8	4-8	AML-M1, M4, M5, MDS, secondary AML
t(15;17)(q22;q12-21)	4-7	AML-M3, M3 variant, DIC
t(1;17)(p36;q12-21)	<1	
del(7)(q22-q36)/-7	2-6	AML, MDS, secondary AML
del(5)(q11-q35)/-5	2-6	AML-M1, M2, MDS, secondary AML
inv(16)(p13q22)	2-7	AML-M4Eo, CNS leukemia
del(16)(q22)	<1	
t(16;16)(p13;q22)	<1	
11q23 rearrangements	2-4	AML-M5a, M5b, M4, secondary AML, high WBC count
del(11)(q23)	1-2	
t(9;11)(p21-22;q23)	1-2	
t(6;11)(q27;q23)	<1	
t(10;11)(p15;q23)	<1	
t(11;17)(q23;q25)	<1	
t(11;19)(q23;p13)	<1	
t(9;22)(q34;q11)	1-3	AML-M1
t/del(12)(p11-p13)	0.5-2	AML-M2, basophilia, MDS
del(20)(q11)	~1	AML-M6, MDS
del(13)(q12-q32)	0.5-1	AML, MDS
inv(3)(q21q26)	0.3-1	AML, thrombocytosis, abnormal megakaryocytes, MDS
ins(3;3)(q26;q2126)	<1	
t(3;3)(q21;q26)	<1	
t(6;9)(p23;q34)	~0,5	AML-M1, M2, M4, basophilia
t(3;5)(q25;34)	0.1-0.5	AML, MDS
t(1;7)(p11;p11)	~0.1	secondary AML, MDS
t(8;16)(p11;p13)	~0.1	AML-M5b, erythrophagocytosis

a. Adapted from references 1-10,14.
b. Various numerical aberrations may occur in AML as primary or secondary abnormalities. Besides +8, -5, and -7 these include +4, +6, +11, +21, +22, and -Y.
c. Frequency of different chromosome abnormalities is age dependent. In children t(8;21), t(15;17), inv16, and 11q23 rearrangements are more common, whereas in adults, above 50 years of age, trisomy 8 and abnormalities of chromosomes 5 or 7 more frequently occur.
Abbreviations used: ins = insertion, CNS = central nervous system. For other abbreviations see text.

between karyotypic aberrations and immunophenotypes of AML will be summarized. In the next two chapters our data on the occurrence of specific immunophenotypes in AML with t(6;9)(p23;q34) and AML with inv16(p13q22) will be discussed.

FEATURES OF AML WITH A SPECIFIC CHROMOSOME ABERRATION

t(8;21)(q22;q22)

The t(8;21)(q22;q22) was the first rearrangement identified in AML (17). This abnormality

is associated with French American British (FAB)-M2, although occasional cases have been referred to other FAB groups , especially AML-M1 (4,6,7,11, 16,18,19). Within the heterogeneous group of AML-M2 patients, about 20% of cases appeared to have t(8;21) (3, 11). Most patients are relatively young with a male predominance (10,11,18, 19). Additional chromosomal abnormalities occur frequently, especially loss of a sex chromosome, trisomy 8, and del(9)(q12q22) (2,7,13,18,19).

TABLE 2. Genes involved in specific chromosome abnormalities in AML.

Chromosome abnormality	Genes rearranged
t(8;21)(q22;q22)	8q22: ?; 21q: *AML1*
t(15;17)(q22;q12-21)	15q: *PML*; 17q: *RAR-α*
11q23 abnormalities	11q23: *MLL/ALL-1*
t(9;22)(q34;q11)	9q: *abl*; 22q: *bcr*
t(6;9)(p23;q34)	6p: *dek*; 9q: *can*

The complete remission (CR) rate is uniformly high (~90%) (1,2,5,6,8,13,16,18,19). Although patients having AML with t(8;21) are generally regarded as having a favorable prognosis, this was not found in all studies (18,19). One study reported a significant association between elevated absolute granulocyte count or white blood cell (WBC) count and poor survival (19). Multivariate analysis revealed that the relatively high survival rates of AML patients with t(8;21) can not be explained by the fact that these patients tend to be younger than other AML patients (5,8).

Typically, the leukemic cells contain a large number of Auer rods and often there is a prominent maturation of cells into the neutrophil lineage (11). Bone marrow eosinophilia is

TABLE 3. Prognostic value of specific chromosome abnormalities in AML[a].

Favorable prognosis	
inv(16)(p13q22)	high response rate and long CR duration
t(8;21)(q22;q22)	high response rate and intermediate CR duration
t(15;17)(q22;q12-21)	intermediate response rate and long CR duration
Intermediate prognosis	
normal karyotype	intermediate response rate and intermediate CR duration
11q23 abnormalities[b]	high response rate and short CR duration
Unfavourable prognosis	
t(9;22)(q34;q11)	low response rate and intermediate survival time
del(5)(q11-q35)/-5	low response rate and short survival time
del(7)(q22-q36)/-7	low response rate and short survival time
+8	high response rate and short survival time
complex abnormalities	

a. Adapted from references 1,3,5,6,8,10,12,13,16.
b. AML having 11q23 abnormalities form a heterogeneous group with respect to prognosis. In general, AML patients with t(9;11) do have a relatively good prognosis, whereas patients with other 11q23 aberrations fare poorly (16).

common (1,11). Immunophenotypic studies in AML patients with t(8;21) demonstrated a heterogeneous immature "granulocytic" phenotype, expressing CD11b, CD13, CD15, CD33, CD34, and HLA-DR (20-24). There is a higher prevalence of terminal deoxynucleotidyl transferase (TdT) positivity in these types of AML (25). Interestingly, in a few reports as well as in our AML series, leukemias with t(8;21) were found to coexpress the CD19 antigen (21-24). In our series seven out of eight AML with t(8;21) were $CD19^+$ (unpublished results). CD19 expression as well as the expression of CD13 and CD33 are relatively weak (Figure 1). By use of double immunofluorescence staining we could demonstrate coexpression of TdT and CD19 at the single cell level in AML patients with t(8;21) (patients N.L., M.L., and F.W. in Chapter 4.2). These results indicate that coexpression of CD19 and TdT is not restricted to precursor B cell differentiation. Whereas 20%-25% of AML-M1 and M2 express the CD7 antigen (26,27), this has not been found in AML with t(8;21) (27).

The proto oncogenes c-*mos* on chromosome 8 and c-*ets*-2 on chromosome 21 have been localized near the breakpoints of t(8;21), but these genes are not structurally affected in these AML and probably they are not involved in leukemogenesis (12,28-32). Recently, a 470 kb yeast artificial chromosome spanning the t(8;21) breakpoint has been isolated (33).

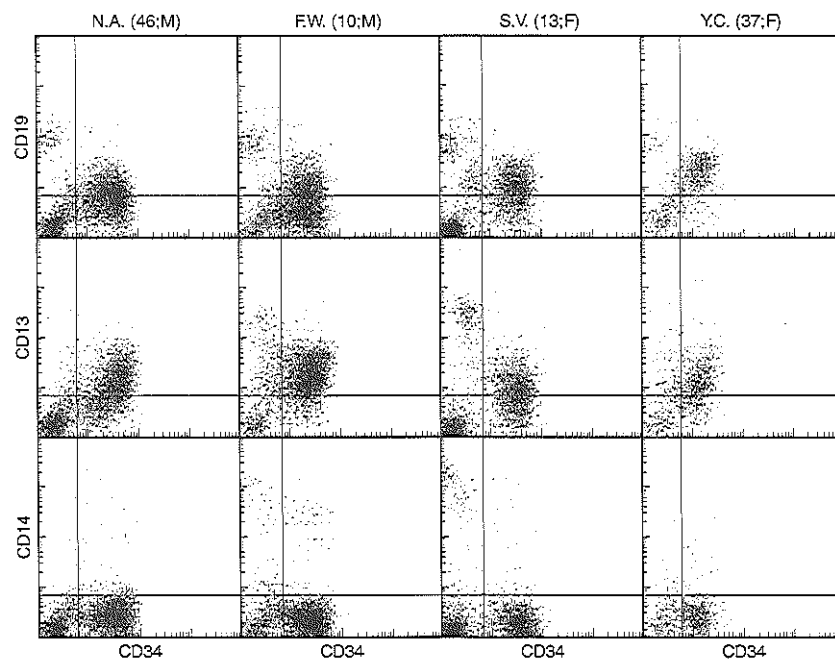

Figure 1. Dot plot analysis of three double IF stainings on PB cells from four different AML patients at diagnosis having AML-M2 with t(8;21)(q22;q22). **Upper:** CD34 (CD34 FITC), CD19 (B4 PE); **Middle:** CD34 (CD34 FITC), CD13 (My7 PE); **Lower:** CD34 (CD34 FITC), CD14 (My4 PE). In addition to a few $CD19^+$, $CD34^-$ B cells it is shown that a part of the $CD34^+$ AML cells is weakly positive for CD19. These $CD34^+$ cells are also weakly $CD13^+$ but $CD14^-$. The CD34 FITC monoclonal antibody was kindly given by Dr. R. Kurrle, Marburg, Germany.

Others have cloned a gene, named *AML*1, in chromosome 21 which was found to be rearranged and transcribed in AML with t(8;21) (34). In the majority of patients investigated the breakpoints are clustered within a limited region of the *AML*1 gene (34). At present it is not known whether rearrangement of the *AML*1 gene results in transcriptional deregulation of the normal gene or in a new fusion gene, which codes for an oncogenic fusion protein.

t(15;17)(q22;q12-21)

Patients with t(15;17)(q22;q12-21) have a typical clinical picture, characterized by young age, low WBC count, decreased platelets, and hemorrhages and/or disseminated intravascular coagulation (DIC) (1,9,35,36). The translocation is highly specific for AML-M3 (4,6,7,11,35,36). Although not detected cytogenetically in 10%-20% of AML-M3 patients, t(15;17) is probably present in all cases with AML-M3. On the other hand, every patient with a t(15;17) probably has AML-M3 or AML-M3 variant (Table 1) (11,35,36). The latter type of AML is characterized by smaller cytoplasmic granules and fewer Auer rods (11,35).

Due to complications at diagnosis, especially hemorrhages, there is a relatively high incidence of early death in patients with AML and t(15;17) (5,16,35). Patients who achieved CR tend to remain in remission for longer periods than average (Table 3) (5,6,15,16,35,36). It is not clear, whether the survival times of AML-M3 cases and AML-M3 variant cases are different.

At the cytogenetic level of resolution, many genes, which are involved in myeloid cells, have been mapped near the breakpoints of t(15;17)(q22;q12-21) (12). These include c-*fes* and the CD13 antigen to chromosome 15q (37), and c-*erb*A1, granulocyte colony stimulating factor (G-CSF), c-*erb*B2, retinoic acid receptor (RAR)-α, and myeloperoxidase (MPO) to chromosome 17q (38-42). The MPO gene is translocated from chromosome 17 to chromosome 15 in AML-M3 patients, but in most cases it was not rearranged (41,43,44). Recent studies could demonstrate that the RAR-α gene is rearranged, whereas the other genes assigned to chromosome 17q11-q22 are not disrupted in t(15;17) (45-50). The translocation breakpoints occur within a 20 kb region in the second intron of the RAR-α gene on chromosome 17q21 and a new transcription unit, called *PML* ("promyelocytes"; initially the term *myl* was used) on chromosome 15q22 (51-54). As a consequence of the translocation, two hybrid fusion genes (*PML/RAR*-α on 17q- and *RAR*-α/*PML* on the 15q+ derivative) are generated (47,49,51-54). In all AML with t(15;17) tested sofar *PML/RAR*-α was found to be actively transcribed, whereas *RAR*-α/*PML* transcripts were found in the majority, but not all, AML-M3 cases (51-54). The resulting chimeric proteins and their role in AML-M3 are currently under investigation (51-53). The *PML/RAR*-α transcript may encode an abnormal receptor molecule, which is involved in the pathogenesis of the disease (52).

Regardless of their pathogenetic significance, *PML/RAR*-α rearrangements have proved to be of value as clonal markers of AML-M3 (54-58). According to recent reports rearrangements of the *RAR*-α locus can be demonstrated in 100% of AML-M3 cases, including cases

with apparently normal karyotypes (55,58,59). Furthermore, these rearrangements enable monitoring of disease status during and after therapy by use of the polymerase chain reaction (PCR) (54,55,57,58,60).

AML with t(15;17) not only exhibits a specific morphology, but also a consistent immunophenotype: positivity for CD9, CD13, and CD33, but negativity for CD15 and HLA-DR (35,61-66). The finding that AML-M3 variant cases are often HLA-DR$^+$ (62), could not be confirmed by others (64-66). Immature markers of AML, such as CD7, CD34, and TdT are usually negative. However, small subpopulations of myeloid marker$^+$, TdT$^+$ cells may be present (67). In one patient such a small TdT$^+$ immature subpopulation was found to be expanded at relapse (67,68). It has been proposed that the CD9$^+$, CD13$^+$, CD33$^+$, CD7$^+$, HLA-DR$^-$ phenotype is specific for all AML-M3 types and might therefore be used for diagnosis of those AML in which cytomorphology is not conclusive (64). However, recently a few AML exhibiting this immunophenotype were reported in which the RAR-α gene was not rearranged (66).

11q23 abnormalities

Rearrangements involving chromosome 11q23 are fairly common in patients with monocytic types of AML, in particular of the immature FAB-M5a subtype. About one half of the patients with AML-M5a have an 11q23 abnormality (4,7,11,69). The 11q23 aberrations represent a heterogeneous group of translocations and deletions (Table 1) (1,10,11). The other chromosome involved in 11q23 translocations is variable, but most common is t(9;11)(p21-22;q23), followed by t(11:19)(q23;p13), t(10;11)(p11-p15;q23), and t(11;17)(q23;q21-25) (Table 1). Deletions of 11q23 have been found in about one third of AML patients with 11q23 aberrations (11). Abnormalities of chromosome 11q23 are frequently observed in congenital and infant AML (70,71) as well as in patients with secondary AML (72-75). Patients with 11q23 abnormalities often have high WBC counts (11).

In general, prognosis for AML patients with an 11q23 abnormality is poor, although differences in outcome have been reported (Table 3) (3,5,13,16). In one study, t(9;11) in *de novo* AML was found to be associated with a superior clinical outcome, while secondary AML with t(9;11) had a poor clinical outcome (76).

Since a wide variety of 11q23 abnormalities, result in monocytic types of AML, it might be hypothesized that a locus on 11q is pathogenetically important. On the other hand 11q23 aberrations occur also in some acute lymphoblastic leukemias (ALL), e.g. t(4;11)(q21;q23) in null-ALL and t(11;19)(q23;p13) in some types of T-ALL (77-79). Initially, rearrangement and amplification of *ets*-1 was found in one case of AML with an 11q23 aberration (80). In two patients with t(11;19) *ets*-1 was shown to move from chromosome 11 to chromosome 19, but the chromosome break did not disrupt the *ets*-1 gene (81). Although it was suggested that the interferon gene cluster on chromosome 9 is rearranged in AML with t(9;11) (82), recent observations suggest that this translocation does not split

this gene cluster (83). Several other genes have been mapped on or near chromosome 11q23, including the CD3, CD56, and *THY1* genes (12,83,84). Recently it was demonstrated that these genes are not disrupted in 11q23 abnormalities and that all breakpoints occur in a 320 kb DNA fragment (83). This locus has been named *MLL* ("myeloid/lymphoid" or "mixed-lineage" leukemia) and two related transcripts could be demonstrated in all normal hematopoietic cells (85). In a cell line with t(4;11) both transcripts were more highly expressed (85). Others have cloned probably an identical gene on chromosome 11q23, named *ALL*-1, in which all AML and ALL breakpoints are clustered (86). The function of the *MLL/ALL*-1 region is unknown.

There are no reports in the literature of an unique immunophenotype of AML with 11q23 changes, which is probably due to the heterogeneous composition of this group of AML. In general, these AML are positive for CD13, CD14, CD33, and HLA-DR, confirming the monocytic phenotype (87-89). However, in a number of cases no expression of the CD14 antigen was found (88). Comparable with some other monocytic types of AML, the CD19 antigen might be expressed (87-90). Moreover, expression of CD7 and CD10 has been demonstrated in AML with q23 abnormalities (90). TdT expression has been found in a few AML cases with an 11q23 aberration (87,89,90). However, in our series all four AML with an 11q23 abnormality [t(9;11) 2x, t(11;19), t(6;11)] were TdT^- (unpublished results), while in other types of AML the majority of cases had a TdT^+ leukemic subpopulation (see also Chapter 4.2). In the study of Gucalp et al., all three patients with del(11)(q23) had a TdT^- AML (91). The observation that the CD56 antigen (gene assigned to chromosome 11q23), is expressed in most monocytic types of AML, but not in AML with an 11q23 abnormality, needs further investigation (92).

Monosomy 5 or 7 and deletion of 5q or 7q

Deletions of chromosomes 5 or 7 or deletions of the long arms of these chromosomes have been found in *de novo* AML, secondary AML, and various types of myelodysplastic syndromes (MDS) or myeloproliferative disorders (1,3,4,11,93-95). In particular, in patients who develop MDS or AML after exposure to radiotherapy and/or cytotoxic chemotherapy abnormalities of chromosomes 5 or 7 are regularly present (93). In contrast to the chromosome abnormalities mentioned above, abnormalities such as -5/del(5q) or -7/del(7q) occur in patients with a median age of over 50 (3,6,93). In childhood, monosomy 7 occurs in a group of patients presenting with a diversity of disorders of myelopoiesis, which all have a poor prognosis (94-96). Consequential, AML with abnormalities of chromosomes 5 and 7 are related to a low response rate to chemotherapy and a short survival. (3,4,5,16,93).

Characteristic features of AML with aberrations of chromosomes 5 or 7 are abnormal megakaryopoiesis, depressed erythropoiesis, and granulocytic abnormalities, including an excess of myeloblasts (11,93). Secondary AML are often difficult to classify, according to the FAB classification, partly because multiple cell lines are involved (11). An association

between CD7 positivity and chromosome 5 aberrations has been described (97). Since aberrations of chromosomes 5 and 7 are relatively common in AML-M6 and AML-M7, erythroid markers and megakaryocytic markers might be positive (98-100). In these types of AML erythroid, megakaryocytic, and myeloid markers are often coexpressed, which is in line with involvement of a multipotent precursor cell (99). AML with monosomy 7 exhibit various (immuno)phenotypes (101,102). In a recent study the only consistent finding was expression of HLA-DR and TdT (102). Using a technique that simultaneously identifies karyotype and immunophenotype, Keinänen et al. could demonstrate multilineage involvement in four patients with monosomy 7 (103). In eight patients with MDS associated with monosomy 7 the karyotypic aberration was not found in lymphoid cells, indicating that it is restricted to committed progenitor cells with the capacity to differentiate into mature myeloid cells (104).

Deletions of the long arm of chromosome 5 are particularly interesting because the deleted region contains a number of genes related to hematopoiesis (see also Table 1 of Chapter 1) (12,88,105-109). These include granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-3, IL-4, IL-5, c-*fms* [=receptor for macrophage-CSF (M-CSF)], and CD14. These genes have been shown to be deleted in the $5q^-$ syndrome (105-109). In addition, genes encoding the glucocorticoid receptor, platelet derived growth factor receptor, endothelial cell growth factor, and β2-adrenergic receptor have been mapped to the long arm of chromosome 5 (109). Initially M-CSF was also mapped to chromosome 5q (110), but recently this gene was reassigned to chromosome 1p13-p21 (111,112). Although the breakpoints and the extent of the deletion of the 5q arm are variable, a region consisting of the 5q31 band is deleted in all patients examined (105,106,108,109). At present, it is not known how the altered growth of hematopoietic cells is caused by deletion of a single allele. An MDS/AML gene has been postulated on chromosome 5q, which functions as tumor suppressor gene, similar to that proposed for retinoblastoma or Wilms' tumor (109,113). The same mechanism might be involved in AML with deletions of chromosome 7 (114). Genes which have been mapped to the 7q21-q31 critical region include the multidrug resistance (MDR) genes (MDR1 and 3), erythropoietin, and plasminogen activator inhibitor type 1 (12,115). However, these genes are not rearranged in myeloid disorders with chromosome 7 abnormalities (12,96,114).

CONCLUSION

Cytogenetic analysis of AML have demonstrated a number of non-random chromosome aberrations. These abnormalities are associated to the biological behaviour of the leukemia, manifested by specific morphological, immunological, and clinical features such as t(15;17), t(8;21), and inv(16). Cytogenetic results in AML have a high prognostic value for prediction of obtaining CR and long term survival. Molecular analysis of breakpoint regions have identified genes which are directly involved in some of the chromosome aberrations,

especially in t(15;17)(q22;q12-21). Most probably other genes involved in AML will be identified in the near future. In AML patients with a well-defined chromosome aberration the PCR technique can be applied to detect minimal disease and thereby to monitor effectiveness of the applied treatment.

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CHAPTER 3.2

TRANSLOCATION (6;9) MAY BE ASSOCIATED WITH A SPECIFIC TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE POSITIVE IMMUNOLOGICAL PHENOTYPE IN AML*

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SUMMARY

Two patients with acute myeloid leukemia (AML) (French American British (FAB)-M4) and t(6;9)(p23;q34) are described. Immunological marker analysis revealed a phenotype of HLA-DR^+, partly terminal deoxynucleotidyl transferase $(TdT)^+$, $CD13^+$ in both cases and CD33 positivity in one. The expression of CD13 and CD33 by TdT positive cells was demonstrated by double immunofluorescence (IF) staining. Although it has been postulated that TdT plays a role in gene rearrangement, Southern blot analysis performed in one leukemia revealed that both the T cell receptor (TcR) β chain genes and the immunoglobulin heavy (IgH) chain genes were in germline configuration. Since we could not detect $CD13^+$, TdT^+ cells and $CD33^+$, TdT^+ cells in control bone marrow (BM) samples, double marker analysis was used to detect low numbers of residual leukemic cells during follow-up of one patient. A gradually increasing percentage of $CD33^+$, TdT^+ cells was detected in the BM in a period of 6 months before hematological relapse. Although the t(6;9) may not be correlated to a specific FAB subtype, it may be associated with TdT positive AML. Since TdT positive AML seems to have a poor outcome, detection of TdT expression in AML patients is particularly important for diagnostic purposes. In addition, our results indicate that double immunological marker analysis for a myeloid marker and TdT allows detection of residual disease during follow-up.

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INTRODUCTION

Specific chromosomal changes, particularly translocations, are associated with specific subtypes of acute leukemia and constitute an independent prognostic factor (1). In myeloid leukemia, there is a good correlation between cytogenetic aberration and morphological subtypes of leukemia as defined by the FAB (2). Such a correlation has only partly been found between immunological phenotypes and FAB morphology (3-5).

In 1976, Rowley and Potter (6) described two patients having AML with t(6;9)(p23;q34). Subsequently, 19 additional cases with the same cytogenetic defect were reported (reviewed in refs. 7 and 8). The morphological diagnoses of these 21 cases were heterogeneous: most were classified as FAB-M2, some as FAB-M4, and a few as FAB-M1, and in at least one-fourth of the cases, a myelodysplastic syndrome (MDS) preceded the onset of the acute leukemia. The immunological phenotypes of these leukemias were not described. We here report on the morphology, immunological phenotype, and cytogenetics of two new AML cases with t(6;9). In addition, in one case the organization of the IgH genes and TcR-β genes was analyzed. The specific immunological phenotype of the leukemic cells of patient 1 was exploited for follow-up studies to detect residual leukemic cells.

CASE REPORTS

Patient 1

A 13-year-old female was admitted in June 1984 with a 6-week history of malaise persisting after an influenza-like illness, together with progressive fatigue, paleness, and loss of weight. Her menses were heavy and persisted for 10 days preceding admission. Physical examination of this pale adolescent girl showed a few petechiae and a just palpable spleen. Laboratory investigations: hemoglobin (Hb) 2.8 mmol/liter, thrombocytes 14 $\times 10^9$/liter, white blood cells (WBC) 29.4 $\times 10^9$/liter with 58% blasts, 2% bands, 32% neutrophils, and 8% lymphocytes. Serum lactate dehydrogenase was 337 U/liter (slightly elevated). BM aspiration showed high normal cellularity with some dysmyelopoietic features. Differential count revealed 56% myeloblasts and 17% smaller not clearly classifiable blasts, 5% promyelocytes, 11% more differentiated neutrophils, 9% normoblasts, and 2% lymphocytes. Neither Auer rods nor an increased number of basophils were seen. Cytochemical stainings showed that 53% of the blast cells were positive with Sudan black, 64% positive with chloroacetate esterase, and 36% positive with α-naphtylacetate esterase but negative after NaF inhibition. An AML, FAB type M4 was diagnosed. Complete remission (CR) was obtained with high doses of cytarabine according to the AML-82 protocol of the Dutch Childhood Leukemia Study Group and maintained with the VAPA-10 maintenance treatment (9). In December 1985 the treatment was electively stopped; in July 1986 she relapsed in the BM. A second remission was obtained with two courses of high doses of cytarabine and asparaginase (10) that was followed by autologous BM transplantation. After another 6 months a second BM relapse occurred, and the patient died.

Patient 2

A 17-year-old female was referred with high fever and extensive candidiasis of the oral cavity. Laboratory investigations: Hb 3.6 mmol/liter, thrombocytes 29 $\times 10^9$/liter, WBC 21.2 $\times 10^9$/liter with 18% blasts. A BM aspirate

was hypercellular with 59% blasts, 7% promyelocytes, 12% more differentiated neutrophils, 10% monocytes, 4% normoblasts, and 8% lymphocytes. In the more mature myeloid cells myelodysplastic features were seen. There were no Auer rods or basophils present. Cytochemical stainings showed that 29% of the blast cells were positive with Sudan black and 23% were positive with α-naphtylacetate esterase but negative after NaF inhibition. An AML, type M4, according to the FAB criteria, was diagnosed. There was no evidence for leukemia infiltration in the gingivae. No pathological lymph nodes were found. Liver and spleen were not palpable and not enlarged on ^{99}Tc scan. She was treated with broad spectrum antibiotics and antimycotic therapy, but no favorable reaction was seen on the temperature or clinical status. Twenty-two days after admission, before cytoreductive treatment was given, she died of gastrointestinal hemorrhage.

MATERIALS AND METHODS

Cytogenetic studies

Cytogenetic studies of BM cells were performed according to our standard procedures including methotrexate treatment of cultures (11). At least 30 cells were analyzed in each instance; the karyotypes were established according to the ISCN (1985) (12).

Immunological marker analysis

Mononuclear cells (MNC) were isolated by Ficoll Paque (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. MNC were incubated with monoclonal antibodies (McAb), directed against B cell antigens, T cell antigens, and myeloid monocytic antigens. Detailed information about the panel of McAb used is summarized in Table 1. As a second step reagent we used a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin (Ig) antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). The expression of TdT was detected by use of a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit Ig antiserum (Supertechs, Bethesda, MD, USA). Double IF stainings for TdT and several surface membrane antigens, i.e., HLA-DR, CD7 (3A1), CD10 (VIL-A1), CD11b (OKM1), CD13 (My7), CD14 (My4), CD15 (VIM-D5), and CD33 (My9), were performed as described (13).

Southern blot analysis

DNA was prepared from BM cells of patient 1 and from control cell samples (14). DNA (15 μg) was digested with the restriction enzymes *Eco*RI or *Bgl*II or by use of a double digestion with *Bam*HI and *Hin*dIII. The digested DNA was size fractionated by electrophoresis on a 0.7% agarose gel and subsequently blotted to nitrocellulose filters. For detection of IgH gene rearrangements the filters with the *Bgl*II and *Bam*HI-*Hin*dIII digested DNA were hybridized to a J_H probe (15). For detection of TcR-β chain gene rearrangements, the filters with the *Eco*RI digested DNA were hybridized to a Cβ probe or a Jβ2 probe (16). Hybridization and washing procedures were performed according to described methods (14).

TABLE 1. Immunological marker analysis of the BM cells from the two patients at diagnosis.

CD[a] (Antibodies)	Antigen recognized	Percentage of positive cells			
		Patient 1		Patient 2	
		Per MNC	Per TdT$^+$ cells[b]	Per MNC	Per TdT$^+$ cells[b]
anti-TdT[c]	TdT	12.5		25.5	
anti-HLA-DR[d]	HLA-DR non-polymorphic antigen	74	26	85	97
CD10 (VIL-A1)[e]	common ALL antigen (CALLA)	0	0	0	0
CD20 (B1)[f]	B cell antigen	1		5	
CD7 (3A1)[g]	Tp41 antigen	4	0	2	3
CD2 (OKT11)[h]	T11 antigen	4			
CD3 (Leu-4)[d]	T3 antigen	1		5	
CD13 (My7)[i]	pan-myeloid antigen	82	89	72	85
CD33 (My9)[i]	pan-myeloid antigen	92	95	0	0
CD11b (OKM1)[h]	C3bi receptor	13	25	3	1
CD15 (VIM-D5)[e]	myeloid antigen	15	0	7	
CD14 (My4)[f]	monocytic antigen	3	0	1	
CD71 (661G10)[j]	transferrin receptor (T9 antigen)	75		85	

a. Cluster of differentiation as proposed by the Workshops on Human Leukocyte Differentiation Antigens (Paris, France, 1982; Boston, MA, USA, 1984; Oxford, UK, 1986; Vienna, Austria, 1989).
b. Percentages positivity for surface membrane marker per TdT positive cells as determined by double IF staining.
c. Supertechs, Bethesda, MD, USA.
d. Becton Dickinson, San Jose, CA, USA.
e. Dr. W. Knapp, Vienna, Austria.
f. Coulter Clone, Hialeah, FL, USA.
g. American Type Culture Collection, Rockville, MD, USA.
h. Ortho Diagnostic Systems, Raritan, NJ, USA.
i. Dr. J.D. Griffin, Boston, MA, USA.
j. Dr. J.M. van de Rijn, Amsterdam, The Netherlands.

RESULTS

Cytogenetic studies

Patient 1.

At diagnosis, 26 of 30 BM metaphases analyzed showed a 46,XX,t(6;9)(p23;q34) karyotype and a 46,XX normal karyotype in four cells (Figure 1). One month later, after induction therapy, the t(6;9) was still present in 2 of 32 metaphases analyzed. Results of subsequent follow-up studies are given in Table 2. At relapse, 25 months after diagnosis, the karyotype showed further clonal evolution, with a main karyotype of 49,XX,+4,6p−,+8,i(9p),+13, in which the 6p− corresponds to the der(6) of the t(6;9), while the der(9) is lost and replaced by an i(9p).

Patient 2.

At diagnosis, 31 of 32 BM metaphases showed the t(6;9)(p23;q34) (Figure 1). All metaphases showed also a constitutional change of chromosome 7, which was paternally inherited.

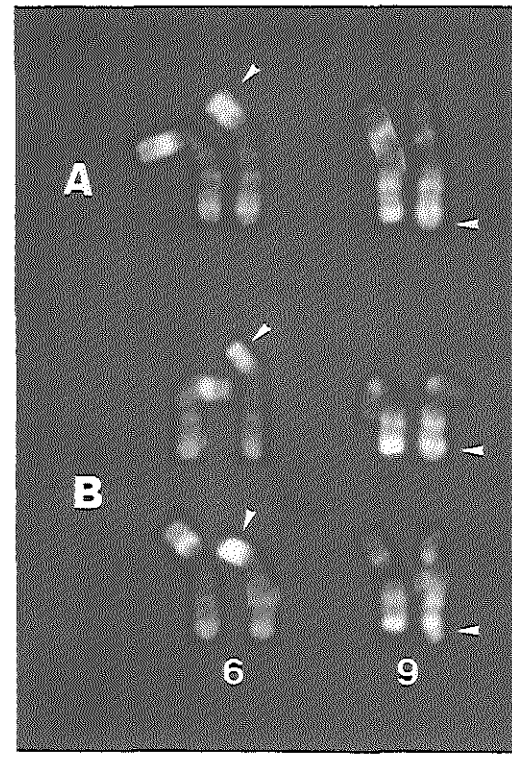

Figure 1. Partial karyotype from BM cells of patient 1 (A) and patient 2 (B) showing t(6;9) (p23;q34). Arrowheads indicate breakpoints. Reverse bands with acridine orange.

Immunological marker analysis

The results of the immunological marker analysis of the BM samples at diagnosis are summarized in Table 1. Most MNC of the BM in both patients were positive for HLA-DR, the transferrin receptor, and the pan-myeloid marker CD13(My7), and in patient 1 also for CD33(My9). TdT was expressed in 12.5% and 25.5% of the MNC of patient 1 and patient 2, respectively. Double IF stainings revealed that in patient 1 most TdT positive cells also expressed CD13(My7) and CD33(My9), and about 25% also expressed HLA-DR and CD11b(OKM1), while the majority of TdT positive cells of patient 2 co-expressed HLA-DR and CD13(My7) (Table 1). Follow-up studies of patient 1 using a double IF staining for CD33(My9) and TdT are given in Table 2. About 0.05% $CD33^+$, TdT^+ cells were found already 4 weeks after maintenance therapy had been electively stopped. The percentage of $CD33^+$, TdT^+ cells increased 10-fold during the next 6 weeks. After another 7 weeks, 17% of the MNC in the BM showed the leukemic phenotype, and 36% of the metaphases showed the t(6;9) with karyotypic evolution. However, morphologically the BM was still in remission. At relapse, which occurred 7 months after discontinuation of therapy, 48.5% of the cells were $CD33^+$, TdT^+ and 90% of the metaphases were abnormal (Table 2).

TABLE 2. Follow-up of patient 1 throughout the course of the disease: cytomorphology, immunological marker analysis, and cytogenetics of the various BM samples.

Date	Clinical phase	% of blast cells	% of $CD33^+$, TdT^+ cells	Karyotype
05 Jun 84	Diagnosis	73	11.7	N(13%)[a]/46,XX,t(6;9)(p23;q34)(87%)
10 Jul 84	CR[b]	4	ND[c]	N(94%)/46,XX,t(6;9)(6%)
08 Oct 84	CR	1	0	N
17 Dec 85	Off treatment	ND	ND	ND
13 Jan 86	CR	0	0.05	N
24 Feb 86	CR	0	0.46	ND
14 Apr 86	CR	3	17.0	N(64%)/47,XX,6p−[d],+8,i(9p)(9%)/ 48,XX,+4,6p−,+8,i(9p)(27%)
10 Jul 86	Relapse	90	48.5	N(9%)/48,XX,+4,6p−,+8,i(9p)(16%)/ 49,XX,+4,6p−,+8,i(9p),+13(75%)

a. N, 46,XX, normal karyotype.
b. CR, complete remission.
c. ND, not done.
d. 6p−, der(6)t(6;9).

Southern blot analysis

Southern blot analysis of DNA from BM cells of patient 1 revealed that both the TcR-β chain genes and the IgH genes were in germline configuration.

DISCUSSION

The two patients described presented with an AML FAB-M4, t(6;9)(p23;q34) in BM metaphases, absence of BM basophilia, and a survival of 25 months and 22 days, respectively. Including these two cases the total number of reported patients having an AML with t(6;9)(p23;q34) is 23: 11 females and 12 males with a median age of 27.5 years (range 5-51) (7,8). As already reviewed by others, AML with t(6;9) is characterized by a poor outcome (11/21 patients showed no or only partial response; 10/21 reached CR of whom at least seven relapsed) and by a heterogenous FAB type association (M2 in 11/22 cases, M4 in 8/22 cases including the two patients reported here, M1 and Ph^1 negative chronic myelocytic leukemia have been reported in two and one cases, respectively) (1,7,8). Other morphological characteristics were proposed, i.e., the absence of Auer rods in FAB-M2 (17) and the presence of BM basophilia, which was found in eight of nine patients by Pearson et al. (18). However, as far as the descriptions are available, in four other cases as well as in our two cases no

increased BM basophilia was seen (7,8). Interestingly, in six of seven cases without basophilia a FAB-M4 diagnosis was made. The presence of MDS features and/or an MDS preceding the onset of acute leukemia is another frequent finding in AML with t(6;9) and was also seen in our two cases (7,18).

As far as we know, this is the first report on the immunological marker analysis of AML with t(6;9). Both leukemias expressed the immunological phenotype HLA-DR$^+$, CD13$^+$, partly TdT$^+$. In addition, the cells of one leukemia were also positive for CD33. Based on positivity for HLA-DR and CD13 and negativity for CD11b and CD14, these two leukemias belong to phenotype group I (i.e., CFU-C) as proposed by Griffin et al. (19). In their study they did not evaluate TdT expression, although several reports indicate that TdT is expressed in 5%-20% of the AML cases (3,4,20-22). Since TdT positive AML as well as AML with t(6;9) are reported to have a poor prognosis, it may be important to evaluate whether TdT positivity is restricted to AML subgroups with a poor response to therapy (18,20,21).

It is notable that in several of the reported TdT positive AML, including our two cases, the percentage of cells positive for TdT is considerably lower than the percentage of leukemic blasts (20,21). In fact, according to our recent observations, TdT positivity may be found in most AML, but often the percentage of these TdT positive cells is low (<10% of MNC) (see Chapter 4.2). This may reflect the presence of leukemic cells in various differentiation stages, a heterogeneity that is often seen in AML (3,4,22-25). Whenever a discrepancy is found between the percentage of TdT positive cells and the percentage of cells positive for a myeloid marker, double marker analysis has to be performed to determine which markers are expressed by the TdT positive cells. Such analyses are described for TdT and myeloperoxidase as well as for TdT and the myeloid antigen CD15 (26,27). In our patients the TdT positive cells did not represent normal early lymphoid cells since they co-expressed myeloid markers like CD13, CD33, and CD11b (Table 1). These TdT positive cells may represent the immature clonogenic subpopulation within the leukemia, which differentiates to more mature TdT negative cells (3,21,22,24,28-30). Most probably the morphological diagnosis is based on the characteristics of the more mature cells, while the so-called immature or not classifiable blasts may represent the TdT positive cells. This phenotypic heterogeneity may explain the absence of a correlation between t(6;9) and a specific FAB subtype. In fact, immature phenotype, poor response rate, and MDS features seem to indicate a stem cell involvement in AML with t(6;9).

Since TdT is postulated to be involved in the insertion of nucleotides during rearrangement of IgH genes and TcR-β genes (31,32), we investigated the configuration of the IgH genes and TcR-β genes in patient 1: both genes appeared to be germline. An abnormal activation of TdT encoding genes due to a chromosomal aberration is not likely since the TdT gene is located on chromosome 10 (33), which showed no visible alteration in both leukemias.

Whether the myeloid marker$^+$, TdT$^+$ phenotype represents a rare myeloid progenitor cell is unknown. Using double IF staining, we investigated the BM cells of 15 patients after withdrawal of maintenance therapy and could not detect any CD13$^+$, TdT$^+$ or CD33$^+$, TdT$^+$ cells (unpublished observations). The data suggested that minimal residual disease could be detected using double IF staining for this specific phenotype. In T cell acute lymphoblastic

leukemia the detection limit of such a double IF staining technique, using a T cell marker and TdT, has proved to be at least 0.01% and allows detection of a relapse 3-5 months before clinical relapse (34). In the case of patient 1, 0.05% of $CD33^+$, TdT^+ cells were found in the BM 4 weeks after stopping the maintenance therapy (Table 2), suggesting the presence of a residual leukemic population. Subsequently, the percentage of $CD33^+$, TdT^+ cells increased gradually. Four months after stopping the therapy, BM was still in remission as defined by morphology (3% blasts) but in relapse by both immunological marker analysis (17% $CD33^+$, TdT^+ cells) and cytogenetic analysis (36% of metaphases with t(6;9) and additional karyotypic changes). Six months after the first detection of $CD33^+$, TdT^+ BM cells and 2 months after the reappearance of the specific karyotypic abnormality, this patient got a morphologically proven BM relapse (Table 2). This illustrates the usefulness of double IF staining for detection of residual disease and for prediction of a relapse in leukemia with a specific phenotype, and it emphasizes the need to perform immunological marker analysis in AML.

In summary, the myeloid $marker^+$, TdT^+ phenotype may be a characteristic feature of AML with t(6;9). Therefore, immunological marker analysis, using double IF stainings, should be performed in other AML with this specific translocation. Furthermore, our data indicate that the myeloid marker, TdT double IF staining is a powerful tool for detection of residual disease and early relapse. This is important in TdT^+ AML since these leukemias generally have a poor outcome.

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CHAPTER 3.3

ACUTE MYELOID LEUKEMIA M4 WITH BONE MARROW EOSINOPHILIA (M4Eo) AND inv(16)(p13q22) EXHIBITS A SPECIFIC CD2$^+$ IMMUNOPHENOTYPE*

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SUMMARY

Extensive immunological marker analysis was performed to characterize the various leukemic cell populations in eight patients with inv(16)(p13q22) in association with acute myeloid leukemia with abnormal bone marrow eosinophilia (AML-M4Eo). Although each AML consisted of heterogeneous cell populations, the immunophenotype of the subpopulations in the eight AML cases was strikingly similar. Virtually all AML-M4Eo cells were positive for the pan-myeloid marker CD13. In addition, the AML were partly positive for CD2, CD11b, CD11c, CD14, CD33, CD34, CD36, CDw65, TdT, and HLA-DR. Double immunofluorescence (IF) stainings demonstrated coexpression of the CD2 antigen and myeloid markers and allowed the recognition of multiple AML subpopulations. The CD2 antigen was expressed by immature AML cells (CD34$^+$, CD14$^-$) and more mature monocytic AML cells (CD34$^-$, CD14$^+$), whereas TdT expression was exclusively found in the CD34$^+$, CD14$^-$ cell population.

The eight AML-M4Eo not only expressed the CD2 antigen, but also its ligand CD58 (LFA (leucocyte function antigen)-3). Culturing of AML-M4Eo cell samples showed a high spontaneous proliferation in all three patients tested. Addition of a mixture of CD2 antibodies against the T11.1, T11.2, and T11.3 epitopes, diminished cell proliferation in two patients with high CD2 expression, but no inhibitory effects were found in the third patient with low frequency and low density of CD2 expression. These results suggest that high expression of the CD2

* *Submitted for publication.*

molecule in AML-M4Eo stimulates proliferation of the leukemic cells, which might explain the high white blood cell (WBC) count often found in this type of AML.

INTRODUCTION

AML is a phenotypically heterogeneous disorder with a marked patient-to-patient variation and intraclonal variation in most patients (1). The French American British (FAB) group has specified different cytomorphological categories, such as AML-M4 with AML-M4Eo (2,3). This FAB category is highly correlated with abnormalities of chromosome 16q22, e.g. del(16)(q22), inv(16)(p13q22), and t(16;16)(p13;q22) (3-8). The 16q22 abnormalities are found in 6% of all AML (5,8-13). Larson et al. suggested that the juxtaposition of DNA regions from 16p13 and 16q22, as occurs in inv(16) and t(16;16), is probably required for the M4Eo phenotype (9). Only a few patients with inv(16) or t(16;16) presented with an other type of AML or with myelodysplastic syndrome (13-15). Initially it was described that the metallothionein gene cluster was split by the chromosome 16 aberration in AML-M4Eo (16). However, recently this gene complex has been remapped to chromosome 16q13 and it was not found to be disrupted in AML-M4Eo (17).

In comparison to other types of AML, patients with AML-M4Eo have a young median age (12,13), they often present with high peripheral WBC count and organomegaly (4,8-10,13), and generally they have a high response rate to induction chemotherapy (4,5,8-12,15). In most studies AML-M4Eo patients have a favorable prognosis (4,5,8-10,12,18), although some reports do not support this (13,15). Relapses in the central nervous system (CNS) occur relatively frequently, generally manifesting as leptomeningeal disease and intracerebral myeloblastomas (8,11,13,15,19).

By use of cytomorphology three different cell types can be recognized within each AML-M4Eo, i.e. blast cells, monocytic cells, and eosinophils (5,13,20). The eosinophils are often dysplastic having abnormal eosinophilic granules, which are admixed with varying numbers of basophilic staining granules (4,5,8,9,11,13,21). They display aberrant positivity for chloroacetate esterase and they often contain PAS positive granules (20). Whether the eosinophilic cells and the monocytic cells in AML-M4Eo represent different lineages is not clear. Morphologically hybrid cells have been described with nuclear folding and chromatin pattern characteristics of monocytes or promonocytes and granules identical to those present in eosinophils (20). Immunological marker analysis, especially double IF staining, is a powerful tool to characterize subpopulations within phenotypically heterogeneous malignancies. Studies on marker expression in AML-M4Eo are scarce and only limited numbers of markers have been tested (21-26).

In this study we performed extensive immunological marker analysis to characterize the various subpopulations in eight cases of AML-M4Eo with inv(16)(p13q22). Various immature and more mature subpopulations were detected in all cases. Although the proportion of each subpopulation varied from patient-to-patient, the composition of all AML-M4Eo was strikingly

similar. A special finding in these eight AML was the expression of the T cell marker CD2 on a part of the leukemic cells.

MATERIALS AND METHODS

Patients and cytomorphology

Four children (<16 years of age) and four adults were diagnosed as having AML-M4Eo (Table 1). In all cases >50% of blast cells were found. The diagnosis of AML-M4Eo was based on cytomorphology of peripheral blood (PB) and bone marrow (BM) smears stained for May Grünwald Giemsa and cytochemistry (Sudan black B, myeloperoxidase, and α-naphlylacetate esterase), according to the revised criteria of the FAB group (3).

Cytogenetic studies

Cytogenetic studies of BM cells were performed according to our standard procedures including methotrexate treatment of cultures (27). At least 30 cells were analyzed on each instance; the karyotypes were established according to the ISCN (1985) (28).

Cell samples for immunophenotyping

PB samples (patients E.K., N.S., A.K., J.M., D.W.) or BM samples (patients E.E., M.B., M.V.) were obtained from the eight AML patients at initial diagnosis. Mononuclear cells (MNC) were isolated by Ficoll Paque (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. These MNC samples were frozen and stored in liquid nitrogen.

Immunological marker analysis

MNC were incubated as described with optimally titrated McAb (29). Several McAb were conjugated with fluorescein isothiocyanate (FITC) or phycoerythrin (PE), which enabled us to perform double marker analysis. We used the B cell markers CD10 (VIL-A1, Dr.W.Knapp, Vienna, Austria), CD19 (Leu-12, Becton Dickinson, San Jose, FL, USA), and CD20 (B1, Coulter Clone, Hialeah, FL, USA); the T cell markers CD2 (T11 and T11 FITC, Coulter Clone; 6G4 (T11.1), 4B2 (T11.2), and HIK27 (T11.3), Dr.R.A.W. van Lier, Amsterdam, The Netherlands), CD3 (Leu-4, Leu-4 FITC, and Leu-4 PE, Becton Dickinson), CD4 (Leu-3 PE, Becton Dickinson), CD7 (3A1, American Type Culture Collection, Rockville, MD, USA), and CD8 (Leu-2 PE, Becton Dickinson); the myeloid monocytic markers CD13 (My7 and My7 PE, Coulter Clone), CD14 (My4, My4 FITC, and My4 PE, Coulter Clone), CD15 (VIM-D5, Dr.W.Knapp; Leu-M1 FITC, Becton Dickinson), CD33 (My9, My9 FITC, and My9 PE, Coulter Clone), and CDw65 (VIM-2, Dr.W.Knapp; BMA-0210 FITC, Prof.Dr.W.Ax, Behring Diagnostica, Marburg, Germany); the erythroid marker glycophorin A (VIE-G4, Dr.W.Knapp); the platelet markers CD42a (FMC25, Dr.H.Zola, Bedford Park, Australia) and CD61 (C17, Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands); the non-lineage specific markers CD11a (TB133, Dr.R.A.W. van Lier; CLB LFA1/2, Central Laboratory of the Blood Transfusion Service), CD11b (CLB mongran/1, Central Laboratory of the Blood Transfusion Service; Leu-15 PE, Becton Dickinson), CD11c (Leu-M5, Becton Dickinson), CD18 (CLB54, Dr.R.A.W. van Lier; CLB LFA1/1, Central Laboratory of the Blood Transfusion Service), CD34 (BI-3C5, Seralab, Crawley Down, UK; HPCA-2 FITC and HCPA-2 PE, Becton Dickinson; CD34 FITC, Dr.R.Kurrle, Behring Diagnostica), CD36 (OKM5, Ortho Diagnostic Systems, Raritan,

NJ, USA), CD54 (BBL-4, British Biotechnology, Oxford, UK), CD58 (TS2/9, Dr.T.Schumacher, Netherlands Cancer Institute, Amsterdam, the Netherlands), and HLA-DR (L243, L243 FITC, and L243 PE, Becton Dickinson). In case of unconjugated McAb we used an FITC-conjugated goat anti-mouse immunoglobulin (Ig) antiserum (Central Laboratory of the Blood Transfusion Service) as second-step reagent. Isotype identical unrelevant McAb, either unconjugated or conjugated with FITC or PE, were used as negative controls. The surface membrane labelings were measured and analyzed by use of a FACScan flow cytometer and FACScan-research software (Becton Dickinson).

The expression of terminal deoxynucleotidyl transferase (TdT) was detected as described by use of a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit Ig antiserum (Supertechs, Bethesda, MD, USA) (29). Double IF staining for TdT and several surface membrane antigens, i.e. CD2 (T11), CD7 (3A1), CD10 (VII-A1), CD13 (My7), CD14 (My4), CD15 (VIM-D5), CD19 (Leu-12), CD33 (My9), CD34 (BI-3C5), CDw65 (VIM-2), and HLA-DR (L243) were performed as described previously (29,30). The binding of the McAb on the surface membrane was demonstrated by use of a tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service). The TdT IF labelings were analyzed on Zeiss fluorescence microscopes (Zeiss, Oberkochen, Germany), equipped with phase contrast facilities (29).

Culture systems

MNC from three of the eight AML (M.B., A.K., J.M.) were cultured. Before culturing T cell depletion was performed by use of CD3 (OKT3; Ortho Diagnostic Systems) and magnetic cell separation (MACS system, Miltenyi Biotec, Bergisch-Gladbach, Germany) as described (31). For all cell culture experiments MNC were adjusted to a final concentration of 5×10^5 cells per ml. Cells were cultured in a serum-free medium as described (32). McAb added in optimal concentrations were: CD2 [a mixture of 6G4 (T11.1), 4B2 (T11.2), and HIK27 (T11.3)], CD18 (CLB54), and/or CD58 (TS2/9). Control cultures did not contain McAb. For measurement of [^{3}H]-thymidine incorporation, MNC were cultured in 96-well flat-bottom tissue culture plates (10^5 cells per well; Costar, Cambridge, MA, USA). Incubation was performed for three days at 37°C, 100% relative humidity, and pCO2 of 5%.

[^{3}H]-thymidine incorporation

MNC were cultured for three days. [^{3}H]-thymidine (specific activity 6.7 Ci/mmol; Amersham International, Amersham, UK) pulsing was for 6h using 0.5 μCi per well. After the 6-h pulse the cells were harvested using an automatic cell harvester (Skatron, Lier, Norway). [^{3}H]-thymidine incorporation was measured with a Betaplate Liquid Scintillation Counter (LKB Wallac, Turku, Finland). Each determination was performed in triplicate.

RESULTS

Cytomorphology and cytogenetics

The eight leukemias were classified as AML-M4Eo. Cytogenetics at initial diagnosis showed an inv(16)(p13q22) in 60% to 100% of the metaphases in the eight cases studied. In patient M.V., a trisomy 22 was seen in 18% of the metaphases in addition to inv(16).

Immunological marker analysis

The results of the immunological marker analysis are summarized in Tables 1, 2, and 3 and in Figures 1, 2, and 3. With the exception of patient E.K., the MNC samples contained >80% of leukemic blasts. The PB MNC sample of patient E.K. enclosed about 10% of CD3$^+$ T cells, about 25% of CD19$^+$ B cells, and about 65% of leukemic blasts. The MNC sample of patient

TABLE 1. Immunological marker analysis of the eight AML-M4Eo[a].

Immunological markers	Patients							
Age;sex Cell sample	E.K. (1;M) PB	E.E. (14;M) BM	M.B. (10;F) BM	N.S. (38;F) PB	A.K. (26;M) PB	J.M. (63;F) PB	M.V. (13;F) BM	D.W. (37;M) PB
Single IF stainings								
CD2 (T11)	50	50	33	34	15	52	51	9
-T11.1 (6G4)	48	42	29	30	17	51	48	10
-T11.2 (4B2)	52	32	24	26	14	49	46	8
-T11.3 (HiK27)	38	17	21	17	5	47	40	5
CD3 (Leu-4)	9	2	3	2	2	3	10	<1
CD4 (Leu-3)		26		23		40		33
CD7 (3A1)		3	2	3	2	8	12	
CD8 (Leu-2)		1		1		2		1
CD10 (VIL-A1)		<1	<1	1	<1	<1	<1	1
CD11a (TB133)	96	98	96	97	99	96	97	99
CD11b (Leu-15)	32	14	18	15	11	30	34	18
CD11c (Leu-M5)		17	19	18	26	43		23
CD13 (My7)	64	95	89	90	96	81	83	97
CD14 (My4)	32	22	20	8	23	34	29	19
CD15 (VIM-D5)	13	20	25	19	11	21	6	9
CD18 (CLB54)	49	71	60	50	80	63	91	79
CD19 (Leu-12)	25	3	1	2	2	2	3	1
CD20 (B1)		3	1	2		3	2	2
CD33 (My9)	40	57	45	60	70	71	67	64
CD34 (BI-3C5)	29	82	65	81	71	41	53	77
CD36 (OKM5)		36		17	23	51		26
CD54 (BBL-4)	49	91	70	90	96	94	83	97
CD58 (TS2/9)	68	95	97	94	93	95	90	87
CDw65 (VIM-2)	22	35	57	36	55	45	56	18
TdT	0.1	40	4	21	0.6	4	7	0.2
HLA-DR (L243)	57	55	35	44	48	60	51	47
Double IF stainings								
CD13$^+$, CD14$^+$	32	22	19	8	23	34	29	18
CD13$^+$, CD34$^+$	27	81		78		41		76
CD14$^+$, CD34$^+$	1	15	12	<1	5	1	8	2
CD33$^+$, CD14$^+$	31	22	18	8	22	34	28	18
CD33$^+$, CD34$^+$	10	45		50		11		41
HLA-DR$^+$, CD14$^+$		17		7	18	34		15
HLA-DR$^+$, CD34$^+$	9	48		32	25	11		30

a. Percentage positivity per MNC.

TABLE 2. Immunological marker analysis of the CD2$^+$ cells[a].

Immunological markers	Patients							
	E.K.	E.E.	M.B.	N.S.	A.K.	J.M.	M.V.	D.W.
CD3 (Leu-4)	18	8	12	7	13	6	20	4
CD13 (My7)	78	94	84	93	83	92	74	95
CD14 (My4)	52	25	32	18	20	58	57	56
CD33 (My9)		62		64	35	94		95
CD34 (HPCA-2)	4	83	68	43	78	13	29	33
HLA-DR (L243)		62		78		92		89

a. Percentages positivity for the various markers per CD2$^+$ cells as determined by double IF staining.

M.V. contained about 10% of CD3$^+$ T cells, whereas the other six AML samples contained <5% of CD3$^+$ T cells (Table 1).

Virtually all AML cells were positive for the pan-myeloid marker CD13. Double IF staining allowed the recognition of immature myeloid cells (CD13$^+$, CD34$^+$) and more mature monocytic cells (CD13$^+$, CD14$^+$) within the CD13$^+$ cell population (Figure 1A and Table 1). In addition, small subpopulations of CD14$^+$, CD34$^+$ and probably also CD14$^-$, CD34$^-$ cells were present (Figure 2C). Only in patient E.E. and M.B. more than 10% CD14$^+$, CD34$^+$ cells were detected (Table 1). In general, the CD14$^+$ cells had the strongest CD13 fluorescence intensity (Figure 1A). In each AML the CD33$^+$ cell population was less prominent than the CD13$^+$ cell population (Table 1). The difference between these two pan-myeloid markers was due to the fact that within the immature CD34$^+$ subpopulation CD33 expression was lower than CD13 expression (Figure 3 and Table 1), whereas the percentages CD13$^+$, CD14$^+$ cells and

TABLE 3. Immunological marker analysis of the TdT$^+$ cells[a].

Immunological markers	Patients							
	E.K.	E.E.	M.B.	N.S.	A.K.	J.M.	M.V.	D.W.
CD2 (T11)	31	78	21	58	27	36	31	48
CD7 (3A1)		1	1	3	0	5	3	0
CD10 (VIL-A1)		0	0	6	0	0	0	0
CD13 (My7)	92	92	66	92	62	65	69	89
CD14 (My4)	0	0	0	2	0	0	0	0
CD15 (VIM-D5)		31	9	61	1		0	
CD19 (Leu-12)			0		0	1	1	
CD33 (My9)	86	81	1	91	20	36	45	56
CD34 (BI-3C5)	100	99	67	97	97	84	81	86
CDw65 (VIM-2)		82		98		80	45	84
HLA-DR (L243)		94	18	96	12		16	23

a. Percentages positivity for surface membrane marker expression per TdT$^+$ cells as determined by double IF staining.

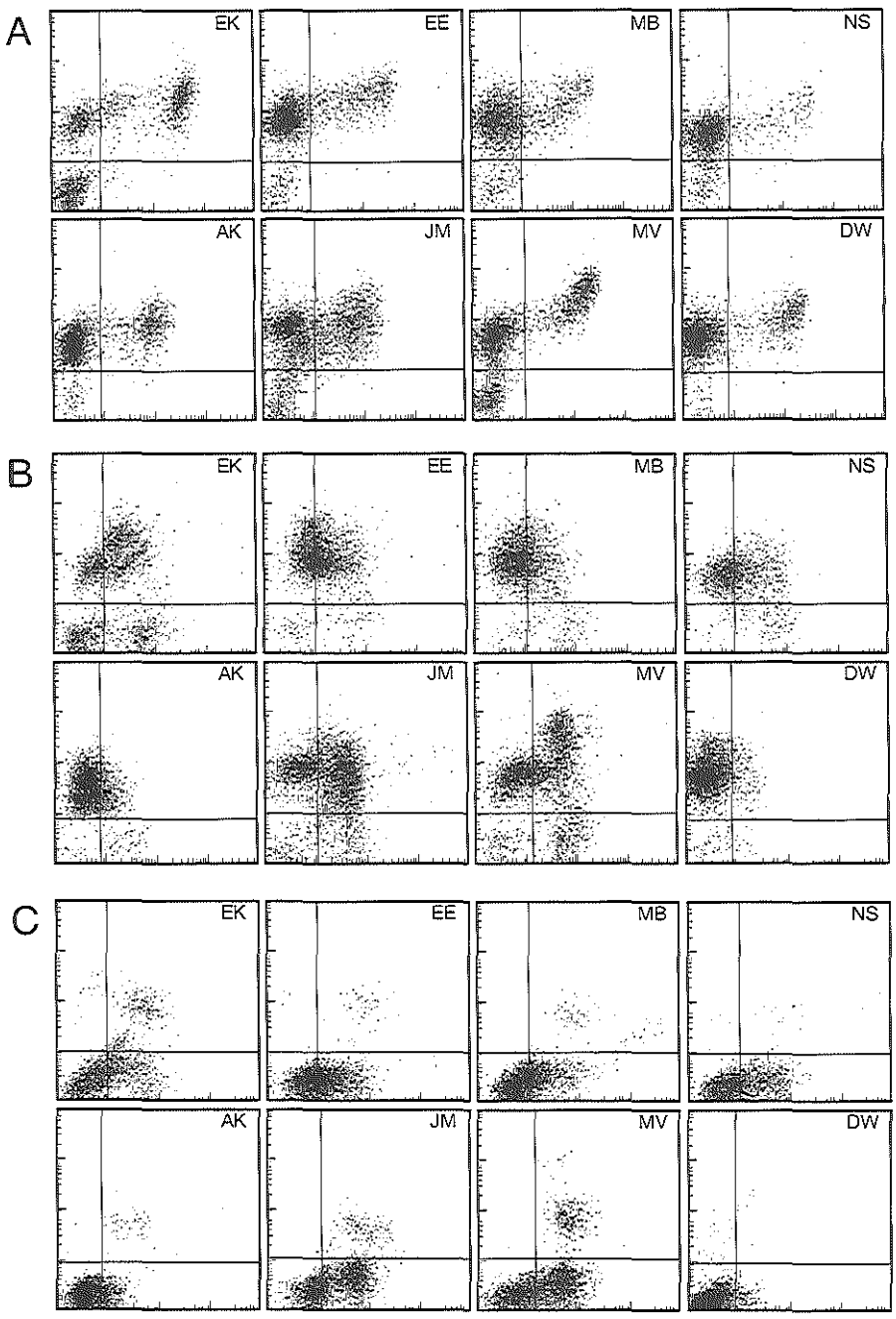

Figure 1. Dot plot analysis of three double IF labelings in all eight AML-M4Eo patients. Green (FITC) fluorescence is shown on the X-asis. Red (PE) fluorescence is shown on the Y-axis. **A:** CD14 (My4 FITC), CD13 (My7 PE); **B:** CD2 (T11 FITC), CD13 (My7 PE); **C:** CD2 (T11 FITC), CD3 (Leu-4 PE).

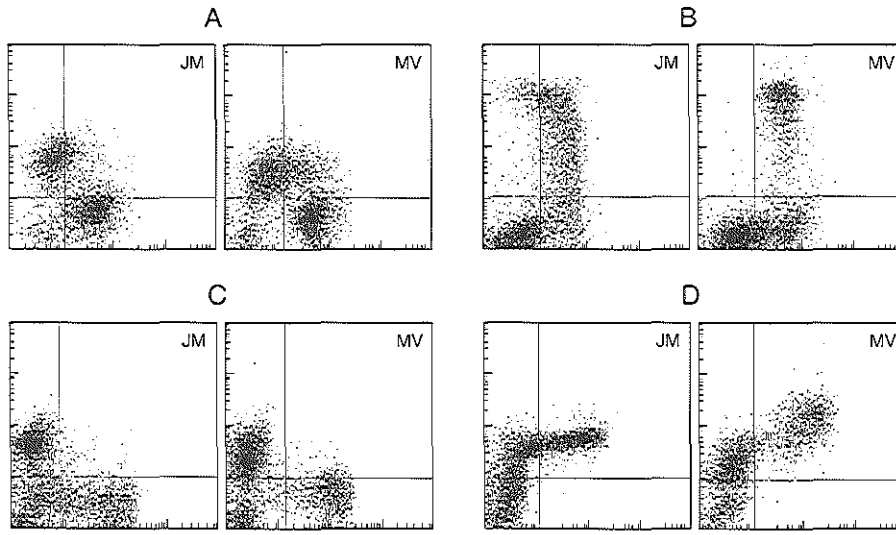

Figure 2. Dot plot analysis of four double IF labelings in patients J.M. and M.V. Green (FITC) fluorescence is shown on the X-asis. Red (PE) fluorescence is shown on the Y-axis. **A:** CD2 (T11 FITC), CD34 (HPCA-2 PE); **B:** CD2 (T11 FITC), CD14 (My4 PE); **C:** CD14 (MY4 FITC), CD34 (HPCA-2 PE); **D:** CD14 (My4 FITC), CD33 (MY9 PE).

$CD33^+$, $CD14^+$ were comparable (Figures 1A, 2D, and Table 1). Although a considerable part of the leukemic cells was $HLA\text{-}DR^+$, a relatively large fraction of the $CD34^+$ cells was $HLA\text{-}DR^-$ (Table 1). Other myeloid markers which were positive on especially the more mature leukemic cells, were CD11b, CD11c, CD15, CD36, and CDw65.

In all eight MNC samples the percentages of $CD2^+$ cells exceeded the CD3 and CD7 percentages (Tables 1 and 2). In Table 2 the results of double IF stainings for CD2 and several other differentiation markers are summarized. In addition to a small $CD3^+$, $CD2^+$ T cell population the majority of the $CD2^+$ cells expressed CD13, CD33, and HLA-DR (Table 2, Figure 1). Despite some patient-to-patient variation, CD2 expression was found in both immature ($CD34^+$) and more mature monocytic ($CD14^+$) leukemic subpopulations (Table 2, Figures 2A, 2B).

Four AML were tested for CD4 and CD8 expression, which revealed weak CD4 reactivity on a part of the leukemic cells. The leukemic cells were positive for the LFA CD11a/CD18 (LFA-1) and its ligand CD54 (ICAM-1) as well as CD58 (LFA-3) (Table 1).

TdT^+ cells were detected in all eight MNC samples. The percentages of TdT^+ cells varied from 0.1% (patient E.K.) to 40% (patient E.E.) (Table 1). The immunophenotype of the TdT^+ cells was determined by use of double IF stainings for TdT and several membrane bound differentiation markers (Table 3). The precursor B cell markers CD10 and CD19 were detected on only a few TdT^+ cells. The TdT^+ cells were positive for HLA-DR and the myeloid markers CD13, CD33, and CDw65 and to a lesser extend for the CD15 antigen. The majority of the TdT^+ cells were $CD34^+$, while only a few $CD14^+$, TdT^+ cells could be detected, indicating that TdT was particularly expressed by the immature AML cells (Table 3).

Culture of AML cells

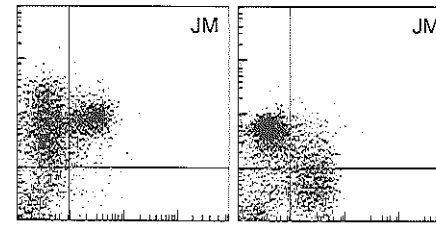

Figure 3. Dot plot analysis of two double IF labelings in patient J.M., showing a lower density CD33 expression on the CD34$^+$ cells as compared to the CD13 expression on these immature cells. Green (FITC) fluorescence is shown on the X-asis. Red (PE) fluorescence is shown on the Y-axis. Left: CD34 (HPCA-2 FITC), CD13 (MY7 PE); Right: CD34 (HPCA-2 FITC), CD33 (MY9 PE).

MNC from three of the eight AML were cultured with or without McAb against LFA (CD2, CD18, and CD58). Due to shortage of cells the culture experiments were performed only once, but each culture was performed in triplicate. Prior to culture the AML samples were depleted of T cells with CD3 McAb using magnetic cell separation. After T cell depletion the percentage of CD3$^+$ T cells in these samples was <1%. The results of the culture experiments are given in Figure 4. High spontaneous proliferation was observed in all three AML. Addition of CD2 McAb to the culture medium inhibited the proliferative response in patients M.B. and J.M., but in patient A.K. the CD2 McAb did not influence the proliferation, which was probably due to the relatively low frequency and low density of CD2 expression in this patient (Figure 1B and Table 1). Addition of CD18 or CD58 McAb did not result in significantly higher proliferative responses in patients M.B. and J.M. However, in patient A.K. addition of CD18 and CD58 McAb or a

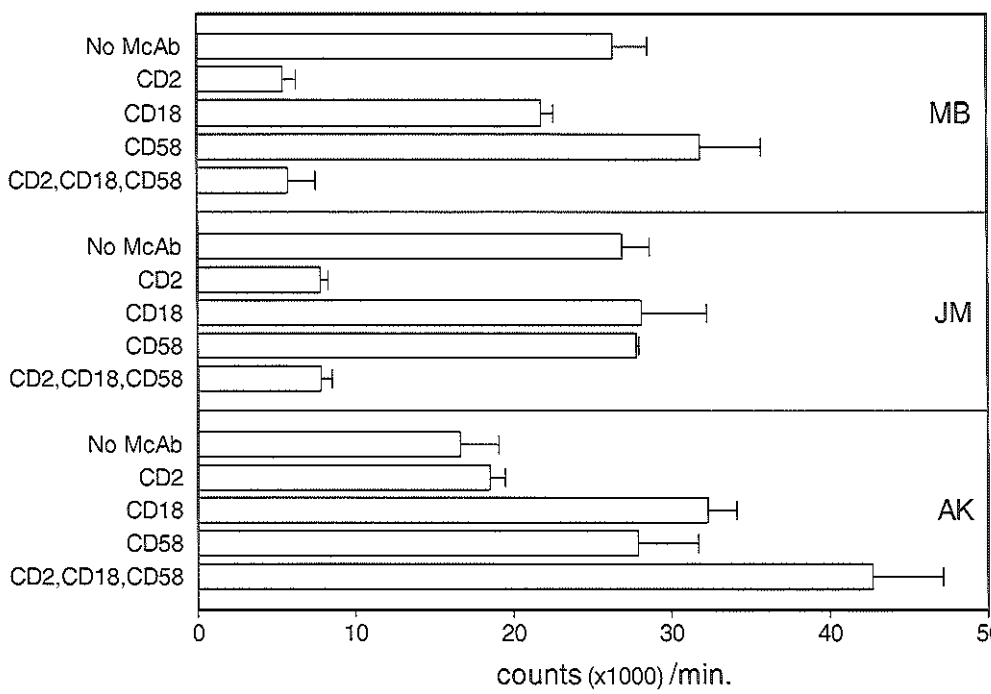

Figure 4. Influence of the addition of CD2, CD18, and/or CD58 McAb upon the *in vitro* poliferation in three AML-M4Eo patients. Each determination was performed in triplate. Results are expressed as mean ± SEM.

mixture of CD2, CD18, and CD58 McAb resulted in an increased proliferative response, probably related to stimulation by the CD18 and CD58 McAb and absence of inhibition by the CD2 McAb (Figure 4).

DISCUSSION

In this study we could demonstrate that AML-M4Eo with inv(16)(p13q22) is associated with a specific $CD2^+$ immunophenotype. Although the eight leukemias consisted of heterogeneous cell populations in which various subpopulations could be identified, the immunophenotype of these subpopulations was strikingly similar. Not only the presence or absence of various immunological markers was comparable, but also the fluorescence intensity of most markers was similar. Whereas a close association between a specific chromosome aberration and a particular (immuno)phenotype is well known in acute lymphoblastic leukemia, this has not frequently been observed in AML, except for the typical phenotype of AML-M3 with t(15;17)(q22;q21), CD19 expression in AML with t(8;21)(q22;q22), and a few associations in other AML types (33-39). This may be explained by the heterogeneous composition of most AML, which can only be characterized properly, if multiparameter analysis is performed (40,41). In addition, small leukemic subpopulations may be missed, if rigid cut-off values of 15% to 25% positivity are used, which is often the case in routine immunological marker analysis.

Virtually all AML-M4Eo cells were positive for the pan-myeloid marker CD13. In addition, the AML were partly positive for CD2, CD11b, CD11c, CD14, CD33, CD34, CD36, CDw65, TdT, and HLA-DR. By use of several double IF stainings we could demonstrate the coexpression of the CD2 antigen and myeloid markers and we could recognize multiple AML subpopulations (Figures 1,2, and 3). Within the $CD13^+$ cell population, immature cells ($CD34^+$, $CD14^-$) and more mature monocytic cells ($CD34^-$, $CD14^+$) could be identified. The CD2 antigen was expressed by immature cells as well as more mature monocytic cells, whereas TdT expression was exclusively found in the $CD34^+$, $CD14^-$ subpopulation. Based on the double IF staining results obtained in our AML-M4Eo cases (Tables 1,2, and 3) a hypothetical diagram of the subpopulations in this type of AML is given in Figure 5.

Reports on immunological marker analysis of AML-M4Eo with a chromosome 16 aberration are scarce and only a minimal number of markers have been used (21-26). If tested, CD13 was found to be positive (21,23). In addition, expression of CD14, CD33, and HLA-DR has been reported, confirming the monocytic phenotype (21,23,25). Paietta et al. described one patient having an AML-M4Eo with inv(16), in whom the cells were $CDw65^+$ and TdT^+ but $CD14^-$ (22). It is not clear whether this leukemia indeed differs from our eight cases or whether the difference can be explained by the presence of a relatively small (<20%) $CD14^+$ monocytic subpopulation, comparable to patient N.S. in our study. Hogge et al. found 20%-25% TdT positivity in two out of six patients with either an inv(16) or a t(16;16) (42). It was not reported whether the other four AML contained <20% of TdT^+ cells (42), as occurs in the majority of TdT^+ AML (30).

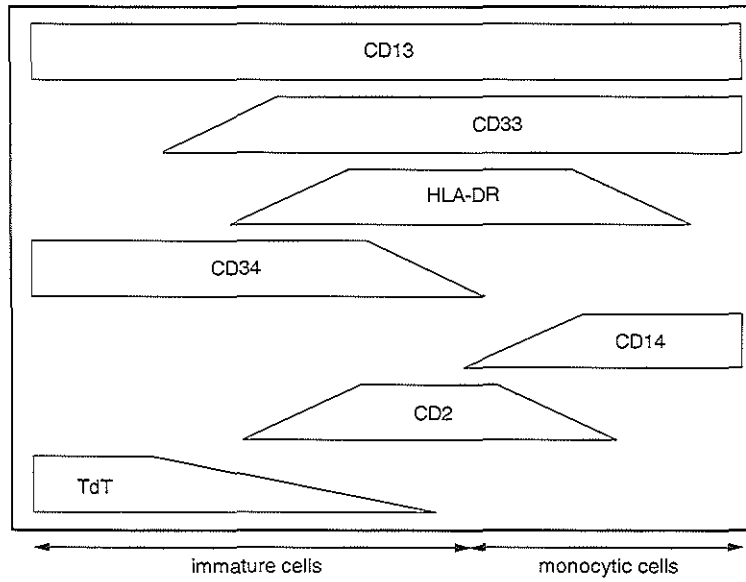

Figure 5. Hypothetical diagram of immunological marker expression by the various leukemic cell populations in AML-M4Eo samples. Virtually all AML-M4Eo cells express CD13. Most cells are positive for either CD34 (immature subpopulation) or CD14 (more mature monocytic subpopulation), whereas CD14$^+$, CD34$^+$ cells and CD14$^-$, CD34$^-$ cells are scarce. CD33, HLA-DR, and CD2 are expressed on a part of the cells in both subpopulations. TdT expression is restricted to the CD34$^+$ subpopulation. This diagram is based on the results of double IF staining experiments, given in Tables 1, 2, and 3.

The consistent expression of the CD2 antigen in our eight AML was detectable by McAb against the three CD2 (T11.1, T11.2, and T11.3) epitopes. In one AML (D.W.) the expression of the T11.1 epitope was confirmed by the ability to form rosettes with sheep red blood cells (data not shown) (43). These results are in line with the report by Ball et al. (26), who demonstrated CD2 mRNA in a case of AML-M4Eo. The negativity for other T cell markers, such as CD3 and CD7, as well as the coexpression of CD2 and myeloid markers argues against T cell lineage commitment (44). According to the literature, CD2 expression can be found in 6%-21% of AML cases (26,45-48). An association between CD2 expression and a specific type of AML has not been reported so far. Schwonzen et al. found that T cell marker positive AML (i.e. CD2, CD4, and/or CD7) often show extramedullary manifestations, especially skin infiltrations (49). The prognostic value of CD2 expression in AML is controversial, since CD2 expression has been associated with both favorable and poor prognosis (26,46). In a recent study in childhood AML expression of the CD2 antigen was not prognostically sifnificant (48). This controversy may be explained by the fact that CD2$^+$ AML form a mixture of AML with a relatively good prognosis (e.g. AML-M4Eo) and AML with poor prognosis, such as immature types of AML and acute undifferentiated leukemias (50).

The CD2 antigen interacts with its ligand the CD58 (LFA-3) molecule, a cell surface glycoprotein with broad tissue distribution including expression on erythrocytes, epithelial cells,

endothelial cells, fibroblasts, and most cells of hematopoietic origin (51,52). Studies in T cells have demonstrated that CD2-CD58 interactions can induce activation in both $CD2^+$ cells and $CD58^+$ cells, leading to proliferation and expansion of the activated T cells (53-55). The AML-M4Eo in our study were not only positive for the CD2 (LFA-2) antigen, but they also expressed the CD58 (LFA-3) antigen. We cultured three AML-M4Eo enabling cell-to-cell contact and observed a high spontaneous proliferation in all three samples. This may explain the reported high success rate of the detection of chromosome 16 aberrations, if cytogenetic analysis is performed on cultured AML-M4Eo cells instead of freshly obtained cells (56). In our culture experiments addition of CD2 McAb inhibited cell proliferation in two patients, suggesting that indeed CD2 (LFA-2)-CD58 (LFA-3) interaction supports the high spontaneous proliferation of the AML-M4Eo cells. However, in the third patient addition of CD2 McAb did not have inhibitory effects on cell proliferation, which might be explained by the low frequency and low density expression of the CD2 antigen in this patient. McAb against LFA molecules, such as CD2, CD18, and CD58, may not only abrogate cell-cell interactions, but they can also function as agonist for signalling through these molecules (43,54,55,57-59). Our data indicate that in at least one patient (A.K.) CD18 and CD58 McAb might indeed induce some additional cell proliferation (Figure 4). In such cases, it is not known whether binding of CD18 and CD58 McAb directly induces cell proliferation or whether this is indirectly caused by the production of cytokines such as interleukin 1 (55).

Finally, it is intriguing to speculate about some unique clinical and biological characteristics of AML-M4Eo, which might be related to the expression of the CD2 and CD58 antigens. The proliferation-inducing effect of the CD2-CD58 mediated cell-cell contact may contribute to the high WBC count in AML-M4Eo (60,61). In addtion, based on the distinct expression of the CD58 antigen on endothelial cells, Plunkett et al. (62) speculated that CD2-CD58 interaction may support extravasation of activated T lymphocytes at sites of immune reaction. Therefore, it is intriguing that patients with AML-M4Eo frequently have enlarged lymph nodes, hepatomegaly, and/or splenomegaly (4,9,10,13). Furthermore, in AML-M4Eo a relatively high incidence of CNS leukemia has been observed, manifesting as leptomeningeal disease and intracerebral myeloblastomas (8,11,13,15,19). Whether expression of the CD2 and CD58 antigens facilitates dissemination of leukemic cells to lymphoid tissues and CNS needs further investigation.

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CHAPTER 4

EXPRESSION OF TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE IN AML: IMPLICATIONS FOR THE DETECTION OF MINIMAL RESIDUAL DISEASE

CHAPTER 4.1

DOUBLE MARKER ANALYSIS FOR TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE AND MYELOID ANTIGENS IN ACUTE MYELOID LEUKEMIA PATIENTS AND HEALTHY SUBJECTS*

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INTRODUCTION

The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed on the nuclear membrane of normal precursor B and T cells as well as their malignant counterparts, i.e., acute lymphoblastic leukemias (ALL) and some lymphoblastic lymphomas (1,2). TdT expression has also been found in 5%-46% of acute myeloid leukemias (AML) (3-9). In AML there is a large variability in the percentage of TdT^+ cells, and also the intensity of TdT expression varies per cell. In most studies a limit of at least 10% of TdT^+ cells was adopted for the diagnosis of a TdT^+ AML. However, it is likely that in some AML smaller TdT^+ leukemic subpopulations are present.

We have analyzed 60 AML for the presence of a TdT^+ subpopulation using double marker analyses for TdT and differentiation markers, such as myeloid markers (CD13 and CD33), B cell markers, and T cell markers. In addition, we applied double marker analyses to monitor the TdT^+ leukemic subpopulation in two AML patients during and after chemotherapy. In control studies, it was investigated whether $CD13^+$, TdT^+ cells and $CD33^+$, TdT^+ cells are present in normal bone marrow (BM) and peripheral blood (PB).

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TABLE 1. Twelve typical examples out of the 60 AML.

Patient	Age	Sex	WBC ($\times 10^9$/liter)	FAB diagnosis	Sample	Blasts (%)	Immunological markers/MNC (per TdT$^+$ cells)[a]											
							TdT	CD2 (T11)[b]	CD3 (Leu-4)[c]	CD7 (3A1)[d]	CD10 (VIL-A1)[e]	CD19 (B4)[b]	CD13 (My7)[b]	CD14 (My4)[b]	CD15 (VIM-D5)[e]	CD33 (My9)[b]	CD34 (BI-3C5)[f]	HLA-DR (L243)[e]
K.A.	60	M	59	M2	PB	82	0	9	9	84	0	2	41	5	3	72	17	56
J.J.	40	M	33	M5b	BM	68	0	13	8	6	0	2	2	37	18	93	3	20
M.S.	0	F	67	M4	BM	41	2	11	8	51 (½)	16 (95)	11	½ (0)	3	14	66 (0)	½	45 (78)
S.M.	16	F	3.5	M6	BM	55	0.1	8 (1)	6	10 (21)	<1 (11)	3 (0)	4 (45)	8 (0)	21 (1)	79 (87)	10 (93)	19 (73)
A.K.	26	M	82	M4	PB	75	0.6	8 (27)	4	2 (0)	0 (0)	3 (0)	95 (62)	20 (0)	11 (1)	80 (20)	67 (97)	48 (12)
P.B.	40	F	13	M2	PB	56	4	4 (0)	2	38 (94)	0 (0)	3 (½)	74 (99)	5 (0)	2 (0)	89 (99)	22 (88)	78 (99)
J.R.	75	M	47	M1	PB	71	6	9 (2)	8	91 (95)	<1 (½)	2 (0)	64 (58)	½ (0)	½ (0)	13 (14)	49 (44)	67 (89)
M.M.	17	M	1.7	M6	BM	71	11	9 (½)	10	10 (5)	<1 (½)	8 (0)	2 (39)	2 (0)	38 (0)	57 (97)	5 (26)	32 (26)
B.B.	13	F	18	M4	BM	73	12	3	½	2 (0)	0 (0)		82 (89)	3 (0)	15 (0)	92 (95)		74 (26)
A.S.	71	M	0.9	M1	BM	88	17	33 (0)	26	23 (0)	0 (0)	0 (0)	36 (99)	½ (0)	1 (0)	14 (54)	39 (99)	3 (0)
P.A.	53	M	47	M3	BM	95	20	½ (0)	2	½ (0)	0		44 (2)	1	0	98 (97)	17 (48)	½ (2)
B.M.	76	F	11	M0	BM	95	65	21 (0)	7	13 (½)	0 (0)	21 (0)	48 (50)	9 (0)	39 (16)	80 (90)	87 (99)	87 (94)

a. Percentages of positivity for a surface membrane marker per TdT$^+$ cells as determined by double IF staining.
b. Coulter Clone, Hialeah, FL, USA.
c. Becton Dickinson, San Jose, CA, USA.
d. American Type Culture Collection, Rockville, MD, USA.
e. Dr. W. Knapp, Vienna, Austria.
f. Seralab, Crawley, UK.

MATERIALS AND METHODS

BM and/or PB samples from 60 patients having an AML were classified according to the revised criteria of the French American British (FAB) group. Immunological marker analyses using fluorescence microscopy were performed on mononuclear cells (MNC) isolated by Ficoll (density, 1.077 g/cm^3) density centrifugation (10). We determined the expression of a series of immunological markers, including TdT, the B cell markers CD10 and CD19, the T cell markers CD2, CD3, and CD7, the myeloid markers CD13, CD14, CD15, and CD33, the HLA-DR antigen, and the precursor antigen CD34. Information about the monoclonal antibodies (McAb) used is given in Table 1. The TdT immunofluorescence (IF) assay was performed by use of a rabbit anti-TdT and a fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit immunoglobulin (Ig) antiserum (Supertechs, Bethesda, MD, USA). Only the characteristic nuclear staining was considered positive. For double marker analysis cells were successively labeled with one of the McAb mentioned above and a tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse Ig antiserum. Subsequently the TdT IF assay was performed. If possible, at least 200 TdT$^+$ cells were analyzed; when MNC samples contained less than 1% of TdT$^+$ cells two cytocentrifuge preparations (total ~50,000 MNC) were screened.

BM and/or PB samples from two patients were monitored for the presence of CD33$^+$, TdT$^+$ cells (patient B.B.) or CD13$^+$, TdT$^+$ cells (patient A.K.) during and after chemotherapy.

Sixty-six BM samples obtained from healthy volunteers (n=7), from ALL patients under therapy (n=14), from ALL patients off therapy (n=45), as well as 25 PB samples from healthy volunteers (n=16) and from ALL patients (n=9) were analyzed for the presence of CD13$^+$, TdT$^+$ cells and CD33$^+$, TdT$^+$ cells.

RESULTS

TdT$^+$ subpopulations in AML at diagnosis

The FAB classification of the 60 AML is summarized in Table 2. Detailed information about hematological characteristics and results of immunological marker analysis of 12 representative AML are given in Table 1. The results of all 60 leukemias will be published elsewhere (11, Chapter 4.2). A marked heterogeneity of marker expression was found between the different leukemias as well as within each leukemia. In 75% (n=45) of the 60 AML a myeloid marker$^+$, TdT$^+$, CD10$^-$ subpopulation was present; this subpopulation varied from 0.1% up to 90% of MNC (Figure 1). In Table 3 the 60 AML are listed according to the size of the TdT$^+$ subpopulation. In most TdT$^+$ AML the TdT$^+$ subpopulation accounted for <50% and often even <10% of MNC. No obvious relationship between the presence or absence of myeloid marker$^+$, TdT$^+$ cells, and the FAB subtypes could be found (Table 2). Within each leukemia the intensity of the TdT expression was variable.

TABLE 2. Presence of myeloid marker$^+$, TdT$^+$ cells in 60 AML

Leukemia type, according to the FAB group	Total number of AML per FAB group	Number of AML with myeloid marker$^+$, TdT$^+$ cells
M1	7	6
M2	22	16
M3	3	3
M4	10	8
M5	8	3
M6	2	2
M0/AUL	8	7

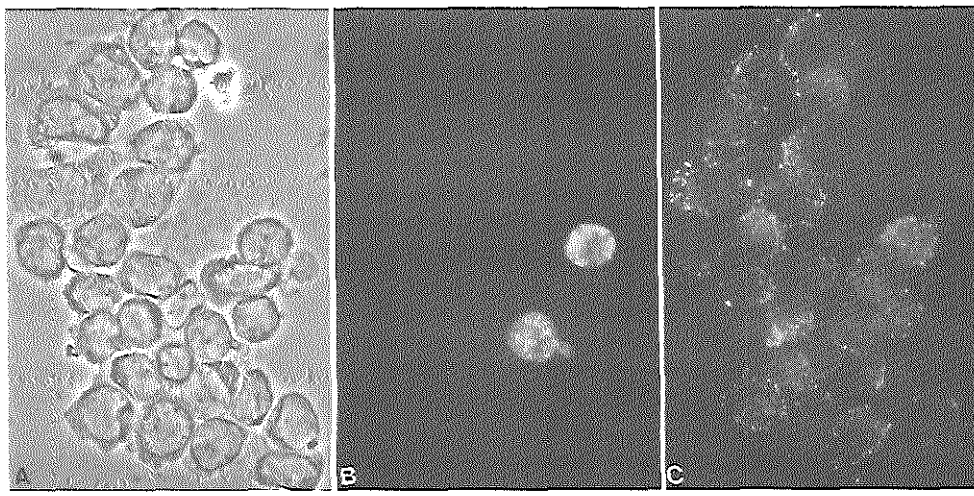

Figure 1. Double IF staining for CD33 and TdT on PB cells from patient P.B. at diagnosis. **A:** Phase contrast morphology; **B:** TdT positive cells (FITC labeled); **C:** CD33 (My9) positive cells (TRITC labeled). The majority of the cells are positive for CD33.

Generally, the TdT expression in AML was weaker than in ALL. In all but one of the 45 AML with a TdT^+ subpopulation the TdT^+ cells were positive for CD13 and/or CD33.

Follow-up of AML patients by use of double marker analysis

Follow-up studies were performed in patients B.B. and A.K. The immunological phenotype of these two AML at diagnosis is given in Table 1, while the follow-up data are summarized in Figure 2.

After stopping the maintenance therapy in patient B.B., a gradual increase in the percentage of $CD33^+$, TdT^+ cells was found during the 6-month period before relapse. After reinduction treatment she obtained complete remission (CR). At this point BM was taken for autologous bone marrow transplantation (ABMT), which was performed 4 weeks later. A second BM relapse occurred 16 weeks after ABMT and the patient died. Although this patient seemed to be in second CR for a period of 20 weeks, in all BM samples tested $CD33^+$, TdT^+ cells were detected (Figure 2).

In patient A.K. both BM and PB samples were monitored. This patient achieved remission after 5 weeks of treatment and obtained CR after 12 weeks. Although at diagnosis only about 1% of the MNC were $CD13^+$, TdT^+, during follow-up low percentages of $CD13^+$, TdT^+ cells were detected in all BM and PB samples tested. The percentages of $CD13^+$, TdT^+ cells gradually decreased, but these double positive cells were still present at week 28 of follow-up. Subsequently the percentages of double positive cells gradually increased to about 5% in the

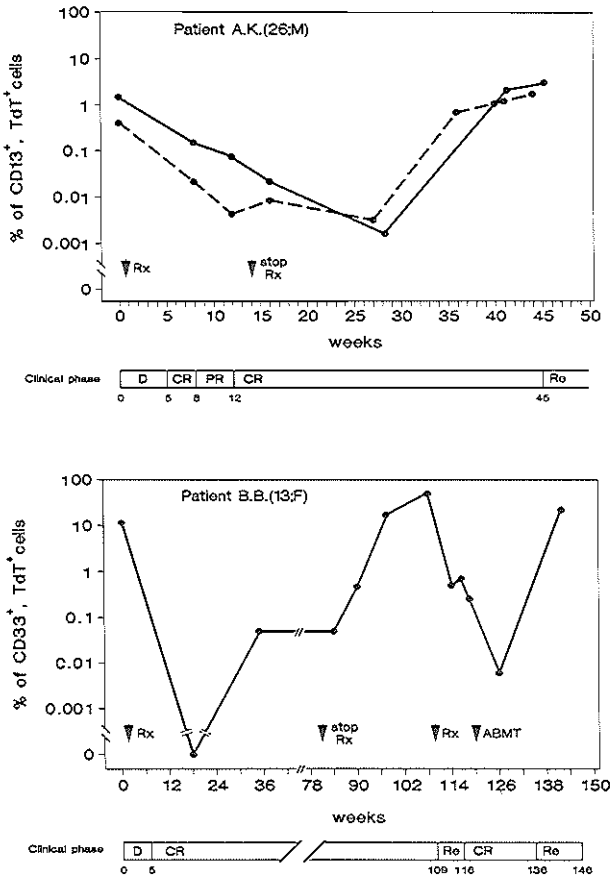

Figure 2. Follow-up of two AML patients by use of double IF staining for TdT and a myeloid marker [CD13 (My7) or CD33 (My9)]. Solid line represents BM and dashed line represents PB. Clinical phase is based on both clinical observation and cytomorphology of BM and PB samples; *D*, diagnostic phase; *CR*, complete remission; *PR*, partial remission; *Re*, relapse. Arrows indicate: *Rx*, start chemotherapy; *Stop Rx*, end of chemotherapy; *ABMT*, autologous bone marrow transplantation.

BM and 3% in the PB at week 45 of follow-up when a BM relapse occurred, as was proven by cytomorphology (Figure 2).

Control studies

Low percentages of myeloid marker$^+$, TdT$^+$ cells (0.001%-0.03%) were detected in 6 out of 7 normal BM samples, in 5 out of 14 BM samples from ALL patients under therapy, and in 4 out of 45 BM samples from ALL patients off therapy (Table 4).

Taken together, in 15 out of 66 (23%) BM samples from healthy volunteers and ALL patients in CR low percentages of CD13$^+$, TdT$^+$ and/or CD33$^+$, TdT$^+$ cells were detected. In 3 of these 15 BM samples both CD13$^+$, TdT$^+$ cells and CD33$^+$, TdT$^+$ cells were present, while in the other 12 BM samples only CD13$^+$, TdT$^+$ cells (n=6) or CD33$^+$, TdT$^+$ cells (n=6) were detected. In general, the nuclear TdT expression as well as the expression of the myeloid

TABLE 3. Percentage of myeloid marker$^+$, TdT$^+$ cells in 60 AML.

Myeloid marker$^+$, TdT$^+$, CD10$^-$ cells (%)	Number of AML	Examples (see Table 2)
0	15 (=25%)	K.A., J.J., M.S.
0.1-10	24 (=40%)	S.M., A.K., P.B., J.R.
10-50	16 (=27%)	M.M., B.B., A.S., P.A.
>50	5 (= 8%)	B.M.

marker was weak.

Myeloid marker$^+$, TdT$^+$ cells were not found in the PB samples (n=25) tested (Table 4).

DISCUSSION

Using double marker analysis for TdT and several differentiation markers, we detected TdT expression in 75% (n=45) of AML. In all cases the TdT$^+$ cells only represented a subpopulation of the AML. Proportionally this subpopulation varied from 0.1% to 83% of MNC. In most cases (n=24) the percentage of TdT$^+$ cells was even <10% (Table 3). The inclusion of such low percentages explains the higher incidence of TdT$^+$ AML in our study as compared with the data reported in the literature (3-9). Nevertheless, our data are comparable with those in other reports. For example, Erber et al. found TdT$^+$ cells in frequencies from 10% to 90% of MNC in 37% of AML cases (8), while we detected such percentages of TdT$^+$ cells in 35% of AML. If a small TdT$^+$ subpopulation (<10%) is present, it has to be demonstrated that these TdT$^+$ cells do not represent normal TdT$^+$ precursor B cells (12). Double marker analysis may allow discrimination between TdT$^+$ precursor B cells and TdT$^+$, myeloid marker$^+$ AML cells (Table 1). Using this approach, it was proven that in the TdT$^+$ AML of our series the TdT$^+$ cells expressed the same pan-myeloid markers as the TdT$^-$ cells (Figure 1).

Since a TdT$^+$ subpopulation occurs in the majority of AML, it is interesting to study whether normal counterparts of such AML cells, i.e., myeloid marker$^+$, TdT$^+$ cells occur in normal BM and PB. Using double marker analysis we found low percentages of CD13$^+$, TdT$^+$ and/or CD33$^+$, TdT$^+$ cells in 23% of the BM samples from healthy volunteers and ALL patients in CR (Table 4). As indicated in Table 4 myeloid marker$^+$, TdT$^+$ cells were detected in the majority of the BM samples from healthy adults, while these cells were found in only 9% of the BM samples from children with an ALL off therapy in continuous CR. The latter finding may be explained by the abundance of CD10$^+$, TdT$^+$ cells, which probably represent regenerating precursor B cells (unpublished observations). Bradstock et al. found about 0.01% of CD13$^+$, TdT$^+$ cells in 5 out of 11 non-leukemia BM samples (13). Interestingly, they detected these double positive cells in BM samples from adults, while these cells were absent in BM samples from young children. Together with our findings these data suggest an age related occurrence of myeloid marker$^+$, TdT$^+$ cells. In addition, Bradstock et al. reported a weak CD13 expression

TABLE 4. Analysis of BM and PB samples from healthy volunteers and non-AML patients for the presence of myeloid marker$^+$, TdT$^+$ cells.

Cell samples	CD13$^+$, TdT$^+$		CD33$^+$, TdT$^+$		Fraction of samples with CD13$^+$, TdT$^+$ cells and/or CD33$^+$, TdT$^+$ cells
	Fraction of positive samples	CD13$^+$, TdT$^+$ cells (%)	Fraction of positive samples	CD33$^+$, TdT$^+$ cells (%)	
BM samples from healthy volunteers aged 18-54 years (n=7)	3/7	0.001-0.03	3/7	0.002-0.02	6/7
BM samples from patients (aged 5-21 years) with an ALL under maintenance therapy in CR (n=14)	4/14	0.002-0.007	4/14	0.004-0.02	5/14
BM samples from patients (aged 5-18 years) with an ALL off therapy in continuous CR (n=45)	2/45	0.02	2/45	0.004	4/45
PB samples from healthy volunteers (n=16) and patients with an ALL in CR (n=9)	0/25	0	0/25	0	0/25

on these cells, which corresponds with our observations for both CD13 and CD33 expression by TdT$^+$ cells in normal BM. In PB samples we did not find myeloid marker$^+$, TdT$^+$ cells. So far, it is unclear whether myeloid marker$^+$, TdT$^+$ cells in normal BM represent precursor myeloid cells or whether the myeloid marker is weakly expressed by precursor lymphoid cells. In this respect, the recent finding of expression of CD13 and CD33 by ALL cells is interesting (14).

The role of TdT in AML cells and in normal precursor myeloid cells is unclear. In precursor lymphoid cells TdT is probably involved in the insertion of nucleotides during rearrangement of Ig genes or T cell receptor (TcR) genes (15,16). In myeloid marker$^+$, TdT$^+$ cells the TdT expression may be related to rearrangement of Ig or TcR genes or to rearrangement of a still unknown myeloid specific gene. On the other hand, aberrant expression of TdT in immature precursor myeloid cells cannot be excluded.

If TdT is expressed by a subpopulation within an AML, double marker analysis can be used to monitor this subpopulation in AML patients during and after chemotherapy. In analogy to our T-ALL studies, we have recently started a follow-up study using double marker analysis for TdT and a myeloid marker in AML patients to detect minimal residual disease (17). Although myeloid marker$^+$, TdT$^+$ cells can be present in low frequencies in BM samples, our preliminary results indicate that detection of minimal residual disease is possible indeed. As illustrated in Figure 2, myeloid marker$^+$, TdT$^+$ cells were detected in two patients, who were in CR on clinical grounds and by cytomorphological examinations of BM and PB samples. Although in

both cases at diagnosis only a subpopulation of the cells were TdT^+, it was possible to monitor this subpopulation during follow-up. In one patient a gradual increase of double positive cells resulted in a hematological relapse after 6 months. In addition, it was also possible to detect myeloid $marker^+$, TdT^+ cells in the autologous BM graft of this patient. The latter suggests that it might be rewarding to search for TdT^+ AML cells in autologous BM grafts of AML patients before transplantation.

In conclusion, TdT^+ cells, from 0.1% up to 83% of MNC, can be detected in the majority of AML. Using double marker analysis it can be demonstrated that these TdT^+ cells belong to the AML cell population. This offers possibilities for the detection of minimal residual disease, and early detection of relapse.

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CHAPTER 4.2

TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE POSITIVE SUBPOPULATIONS OCCUR IN THE MAJORITY OF AML: IMPLICATIONS FOR THE DETECTION OF MINIMAL DISEASE*

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SUMMARY

A series of 60 acute myeloid leukemias (AML) was analyzed for the expression of terminal deoxynucleotidyl transferase (TdT). The detected TdT^+ cells were studied in detail by use of double marker analyses for TdT and differentiation markers, such as myeloid markers (CD13 and CD33), B cell markers, T cell markers, and the precursor antigen CD34. In 15 (25%) of these leukemic cell samples, we found no TdT^+ cells or low percentages of $CD10^+$, TdT^+ cells; the latter probably represent precursor B cells. In the other 45 (75%) AML myeloid $marker^+$, TdT^+, $CD10^-$ cells were detected, ranging from 0.1%-10% (n=24) or over 10% (n=21) of mononuclear cells (MNC). Interestingly, a higher frequency of CD34 positivity was found on the TdT^+ cells as compared to the TdT^- cells, suggesting that the TdT^+ cells represent an immature leukemic subpopulation. Therefore, it may be speculated that the TdT^+ subpopulation contains the clonogenic AML cells. In two patients, in whom immunological marker analysis was performed at initial diagnosis as well as at relapse, an expansion of the TdT^+ subpopulation was documented at relapse, which may reflect a reduced differentiation capacity of the leukemic cells. Previous studies on a series of non-leukemic bone marrow (BM) and peripheral blood (PB) samples revealed that normal counterparts of myeloid $marker^+$, TdT^+ cells are rare in BM (< 0.03%, if they occur at all) and that such cells are not detectable

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in PB. Therefore myeloid marker, TdT double stainings may be useful to monitor the TdT^+ leukemic subpopulation in patients with a TdT^+ AML during and after chemotherapy. Our preliminary results on the follow-up of two such patients support this hypothesis.

INTRODUCTION

TdT is an enzyme which is probably involved in the insertion of nucleotides during rearrangement of immunoglobulin heavy chain (IgH) genes and T cell receptor β chain (TcR-β) genes (1-3). It is found on the nuclear membrane of normal precursor B and T cells as well as their malignant counterparts, i.e., acute lymphoblastic leukemias (ALL) and some lymphoblastic lymphomas (4,5). The expression of TdT by almost all ALL and the majority of the lymphoblastic lymphomas makes TdT very suitable as a marker for these malignancies at diagnosis as well as during follow-up (4-6).

TdT positivity is also found in some AML (7-16). In contrast to ALL, a great variability in the number of TdT positive cells is observed and the intensity of TdT expression per cell is usually lower (9,11-13,15). Initially about 10% of AML was thought to be TdT^+ (7,8), but in some recent reports percentages between 20% and 46% are mentioned (9,12-14). These differences may be explained by the sensitivity of the immunocytochemical procedure used and the types of AML under study as well as the percentage of TdT^+ cells which is accepted as the lower limit for the diagnosis of a TdT^+ AML (9,11,13-15). In most recent studies this lower limit was 10% (10,11,13-15), but it is likely that in some AML a smaller TdT^+ subpopulation is present.

We have analyzed 60 AML for the presence of a TdT^+ subpopulation using double marker analyses for TdT and differentiation markers, such as myeloid markers, B cell markers, and T cell markers. It was found that in the majority of AML a TdT^+ leukemic subpopulation can be detected. In addition, we applied double marker analysis to monitor the TdT^+ leukemic subpopulation in two AML patients during and after chemotherapy.

MATERIALS AND METHODS

Patients

PB and/or BM cells from 60 consecutive AML patients were investigated; 17 patients were children (< 18 years). Patients with a myeloid blast crisis of a chronic myelocytic leukemia or patients who were known with a preceding myelodysplastic syndrome were not included. In six patients the analyses were performed at first relapse (n=5) or at second relapse (n=1). The diagnosis of AML was based on cytomorphology of PB and, when available, BM smears stained for May Grünwald Giemsa and cytochemistry (Sudan black B, myeloperoxidase and α-naphtylacetate esterase). The cases were classified according to the revised criteria of the French American British (FAB) group (17).

Immunological marker analysis

MNC were isolated from BM or PB by Ficoll Paque (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. MNC were incubated with monoclonal antibodies (McAb) as described (18). Several McAb were used, including the B cell markers: CD19 (B4; Coulter Clone, Hialeah, FL, USA) and CD10 (VIL-A1; Dr. W. Knapp, Vienna, Austria); the T cell markers: CD2 (T11; Coulter Clone), CD3 (Leu-4; Becton Dickinson, San Jose, CA, USA), and CD7 (3A1; American Type Culture Collection, Rockville, MD, USA); the myeloid monocytic markers: CD13 (My7; Coulter Clone), CD14 (My4; Coulter Clone), CD15 (VIM-D5; Dr. W. Knapp), and CD33 (My9; Coulter Clone); the non-lineage specific markers: CD34 (BI-3C5; Seralab, Crawley Down, UK) and HLA-DR (L243; Becton Dickinson). In addition, a few leukemias were tested with the myeloid marker CDw65 (VIM-2; Dr. W. Knapp). As a second step reagent we used a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse immunoglobulin (Ig) antiserum (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). A leukemia was regarded to be positive for an immunological marker if more than 20% of MNC expressed the marker. However, in the CD2 and CD7 expression, we decided that the percentages positivity had to exceed the CD3 expression more than 20% because $CD3^+$ T lymphocytes also express CD2 and CD7. The expression of TdT was detected by use of a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit Ig antiserum (Supertechs, Bethesda, MD, USA). Only the characteristic nuclear TdT staining was taken as positive. Double marker analyses for TdT and several surface membrane antigens, such as CD2, CD7, CD10, CD13, CD14, CD15, CD19, CD33, CD34, CDw65, and HLA-DR were performed as described (18). In short: MNC were labeled with a McAb and a tetramethyl-rhodamine (TRITC) conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service). The labeled cells were spun onto slides by use of a cytocentrifuge (Nordic Immunological Laboratories, Tilburg, The Netherlands) and subjected to the TdT staining. No cross-reactivity between the goat anti-rabbit Ig antiserum and the applicated McAb was observed. If possible, at least 200 TdT^+ cells were analyzed; when MNC samples contained less than 1% of TdT^+ cells, two cytocentrifuge preparations (total ~ 50,000 MNC) were screened. The immunofluoresence (IF) labelings were analyzed on Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany), equipped with phase contrast facilities (18).

Follow-up of two AML patients

BM samples from two AML patients (M.M. and B.B.) were monitored for the presence of $CD33^+$, TdT^+ cells during and after chemotherapy.

RESULTS

Fifty-nine leukemias were classified as acute myeloblastic leukemia (AML), and one leukemia was minimally differentiated, lacked myeloid marker expression and therefore classified as acute undifferentiated leukemia (AUL). In Table 1 the leukemias are listed according to their FAB diagnosis. Age, sex, white blood cell (WBC) count, percentage of blast cells in BM and PB, hemoglobin (Hb) level, platelet count of each patient, as well as FAB diagnosis of each leukemia are shown in Tables 2, 3, and 4.

TdT expression

TdT^+ cells were demonstrated in the majority (47 of 60) of the cell samples. The percentage of TdT^+ cells varied from 0.1%-83%. The intensity of the TdT expression on the nuclear membrane of the blast cells varied from leukemia to leukemia. In general, the TdT staining was weaker than the TdT staining in ALL. Myeloid $marker^+$, TdT^+ cells could be demonstrated in 75% of the AML. These double positive cells had a myeloid appearance in phase contrast morphology and were negative for the lymphoid marker CD10. No obvious relationship between the presence or absence of myeloid $marker^+$, TdT^+ cells and the FAB subtypes could be found, except for FAB-M5 in which subgroup only a minority of the leukemias (3 of 8) contained such double positive cells (Table 1). In Tables 2, 3, and 4, the leukemias are listed according to the frequency of myeloid $marker^+$, TdT^+ cells. In Table 2, 15 leukemias are grouped in which no myeloid $marker^+$, TdT^+ cells were found; two AML BM samples contained 2.1% (patient M.S.) and 0.15% (patient J.W.) TdT^+ precursor B cells. The latter was demonstrated by use of double marker analysis, which revealed that the TdT^+ cells in the BM samples from patient M.S. and patient J.W. were $CD10^+$ and myeloid $marker^-$. Data about leukemias with a myeloid $marker^+$, TdT^+ subpopulation are summarized in Table 3 (0.1%-10% TdT^+ cells) and Table 4 (>10% TdT^+ cells).

TABLE 1. Presence of myeloid $marker^+$, TdT^+ cells in 60 AML

Leukemia type according to the FAB group	Total number of AML per FAB group	Number of AML with myeloid $marker^+$, TdT^+ cells
M1	7	6
M2	22	16
M3	3	3
M4	10	8
M5	8	3
M6	2	2
M0/AUL	8	7

Single immunological marker analysis

The results of the immunological marker analysis of the leukemias are summarized in Tables 2, 3, and 4. With the exception of one leukemia (patient H.B.), positivity for CD13 and/or CD33 was found in all leukemias. The majority of the leukemias was positive for HLA-DR, while in some leukemias no or only low percentages of HLA-DR positive cells were demonstrated. The $HLA\text{-}DR^-$ leukemias mainly belonged to the FAB subgroups M1, M2, and M3. Expression of CD14 seems to be restricted to AML-M4 and AML-M5, although CD14 positivity was also found in two AML-M2 (patients N.L. and M.W.). CD15 expression was found in some AML-M4 and AML-M5, but this antigen was also expressed by some other leukemias (e.g., the AUL from patient H.B., the AML-M0 from patient B.M., and the two AML-M6 from patients S.M. and M.M.). CD7 expression was found on 11 "more immature" leukemias (i.e., AML-M0, M1, M2, and M4). CD34 was expressed by most leukemias tested; the percentages

TABLE 2. Fifteen AML without myeloid marker$^+$, TdT$^+$ cells.

Patients	Age	Sex	WBC ($\times 10^9$/liter)	Blasts BM (%)	Blasts PB (%)	Hb (mmol/liter)	Platelets ($\times 10^9$/liter)	FAB diagnosis	Immunological marker analysis per MNC (per TdT$^+$ cells)[a]											
									TdT	CD2	CD3	CD7	CD10	CD19	CD13	CD14	CD15	CD33	CD34	HLA-DR
K.A.	60	M	59	78	82	5.2	14	M2	PB	0	9	9	84	0	2	41	5	3	72	17	56
A.B.	0	M	72	73	65	4.9	18	M5	PB	0	7	8	8	½	4	70	32	66	68		84
J.B.	62	M	51	81	21	5.0	41	M1[b]	PB	0	2	1	5	0	0	18	9	19	58	93	1
R.H.	5	M	5.4	ND[c]	34	7.1	14	M5	PB	0	22	29	20	½				68	65		78
J.J.	40	M	32	68	10	6.0	57	M5b	BM	0	13	8	6	0	2	2	37	18	93	3	20
J.M.	68	F	195	28	92	6.3	36	M5b	PB	0	3	0	1	0	4	50	71	17	82		94
H.P.	41	F	55	ND	96	5.7	27	M2[b]	PB	0	5	5	4	0	½	59	0	½			
P.R.	0	M	4.0	32	21	3.6	10	M4	PB	0		21	22	0		0	69	60	68		74
P.M.	11	M	87	98	81	7.6	32	M0	BM	0	5	2	5	½	3	0	2	0	97	3	96
C.M.	34	F	83	96	80	6.0	34	M2	PB	0	5	4	6	0	2	59	1	8	91	3	42
P.V.	68	M	3.8	46	33	4.5	21	M2	PB	0	12	11	10	0	2	2	1	6	86	2	71
A.R.	73	F	3.3	79	26	4.0	13	M2	BM	0	6	1	1	0	2	1	0	3	84	0	0
Z.H.	58	F	352	90	88	5.8	152	M2	PB	0	2	1	84	0	½	1	1	5	94	2	85
J.W.	0	M	91	79	79	6.0	175	M5	BM	0.15	17	28	29	3(99)	6	23	61	37		38	71
M.S.	0	F	67	41	53	3.7	14	M4	BM	2.1	11	8	51(½)	16(95)	11	½(0)	3	14	66(0)	½	45(78)

a. Percentages positivity for surface membrane marker per TdT positive cells as determined by double IF staining.
b. Analyses performed at first relapse.
c. ND: not determined

TABLE 3. Twenty-four AML with 0,1%-10% of myeloid marker$^+$, TdT$^+$ cells.

Patients	Age	Sex	WBC ($\times 10^9$/liter)	Blasts BM (%)	Blasts PB (%)	Hb (mmol/liter)	Platelets ($\times 10^9$/liter)	FAB diagnosis	Immunological marker analysis per MNC (per TdT$^+$ cells)[a]												
										TdT	CD2	CD3	CD7	CD10	CD19	CD13	CD14	CD15	CD33	CD34	HLA-DR
C.T.	70	M	20	ND[b]	56	5.0	81	M0	PB	0.1	3(2)	3	77(5)	0(0)	½(0)	83(92)	10(0)	5(0)	62(0)	86(63)	83(68)
S.M.	16	F	3.5	55[c]	3	5.8	34	M6	BM	0.1	8(1)	6	10(21)	<1(11)	3(0)	4(45)	8(0)	21(1)	79(87)	10(93)	19(73)
N.L.	52	M	83	56	52	7.2	25	M2	PB	0.2		6	5(0)	0(0)	21(90)	20(84)	44	25(0)	51(8)		48(88)
H.R.	79	F	3.5	83	74	5.8	44	M1	BM	0.3	7(0)	5	6(3)	<1(½)	2(1)	79(70)	2(0)	½(0)	64(11)	56(69)	47(54)
M.K.	58	F	180	86	96	4.7	22	M2	PB	0.3	3(0)	3	2(0)	0(0)	3(0)	2(0)	8(0)	1(0)	89(74)	0(0)	10(3)
A.H.	67	M	2.8	50	16	4.3	110	M5a	BM	0.5	(0)	8	9(0)	½(25)	0(0)	75(70)	15(0)	10(8)	87(70)	33(99)	52(14)
A.K.	26	M	82	90	75	6.2	38	M4	PB	0.6	8(27)	4	2(0)	0(0)	3(0)	95(62)	20(0)	11(1)	80(20)	67(97)	48(12)
L.H.	77	M	1.3	75	0	3.9	16	M1	BM	1.4	0(0)	5	5(0)	1(6)	3(0)	76(65)	2(0)	5(0)	0(0)	30	11(½)
A.G.	71	F	49	68	80	4.0	41	M4	PB	3	13(0)	9	11(21)	0(0)	2(0)	77(81)	6(0)	0(0)	17(13)	61(86)	88(81)
R.P.	6	F	40	88	79	1.6	11	M3	BM	3	8	4	4(½)	0		51(3)	0	2	29(94)	6(69)	17(2)
W.V.	25	M	39	56	78	4.4	43	M2	BM	3	2(½)	2	1(½)		0(0)	53(55)	1(0)	1(0)	92(99)	66(23)	4(0)
M.L.	7	F	10	64	62	6.8	54	M2[d]	BM	4	6(0)	4	7(½)	<1(1)	56(88)	38(5)[d]	2(0)	17(16)	56(2)[d]	57(92)	48(65)
D.N.	9	F	1.3	78	1	6.0	12	M3	BM	4				0		10(9)	11		45(91)		13
M.B.	10	F	77	79	90	6.7	6	M4	BM	4	8(21)	4	2(1)	0(0)	1(0)	75(66)	26(0)	27(9)	45(1)	57(67)	35(18)
P.B.	40	F	13	70	56	7.3	16	M2[e]	PB	4	4(0)	2	38(94)	0(0)	3(½)	74(99)	5(0)	2(0)	89(99)	22(88)	78(99)
M.G.	18	F	104	86	59	5.4	74	M2	BM	5	0(0)	0	18(11)	0(0)	2(0)	70(30)	19(0)	½(0)	73(4)	14(4)	53(7)
J.L.	64	F	27	95	89	3.8	25	M2	PB	5	2(1)	1	½(½)	0(0)	½(1)	0(0)	0(0)	1(0)	86(99)	0(0)	½(0)
B.W.	46	M	1.6	ND	92	5.6	137	M0	BM	6	11(0)	11	90(99)	0(0)	6(1)	1(0)	0(0)	1(0)	42(11)	77(80)	61(70)
E.C.	72	F	92	95	89	5.0	83	M0	BM	6	4	4	4(1)	0(0)	2	93(81)	2	2	<1(1)	30	83(25)
C.K.	68	F	5.3	68	57	4.2	39	M2	BM	6	15	12	10	0		33(83)	8(0)	6(0)	70(6)		62(96)
J.R.	75	M	47	ND	71	3.9	12	M1	PB	6	9(2)	8	91(95)	<1(½)	2(0)	64(58)	½(0)	½(0)	13(14)	49(44)	67(89)
M.V.	13	F	6.5	57	31	7.4	158	M4	BM	7	46(31)	8	17(3)	0(0)	3(1)	81(29)	38(0)	6(0)	76(45)	55(81)	60(16)
R.S.	18	M	150	97	95	4.2	81	M0	PB	7	1(½)	1	77(54)	0	3	0(0)	0	1	96(28)	0(0)	83(80)
L.D.	20	M	105	89	77	6.9	103	M4[e]	PB	9	4(1)	½	29(15)	0(0)	0	21	1(0)	4(0)	65(9)	25(96)	99(96)

a. Percentages positivity for surface membrane marker per TdT positive cells as determined by double iF staining.
b. ND: not determined.
c. Including erytroblasts.
d. CDw65 was found to be positive: 49% per MNC and 84% per TdT$^+$ cells.
e. Analyses performed at first relapse.

of CD34 positivity varied from 0.5%-93%. CD2 and CD19 were only expressed by two and three AML, respectively. CD10 was negative on all cases, with the exception of low percentages $CD10^+$ cells in some cell samples; these cells probably represent precursor B cells.

Immunological marker analysis of TdT^+ cells

The results of the double marker analysis, i.e., the percentages of TdT^+ cells which express a certain differentiation marker, are shown in Tables 2, 3, and 4. Some differences were observed between marker expression by the TdT^+ cells as compared with the expression of those markers by the total population of MNC.

With the exception of the AUL mentioned above (patient H.B.), expression for CD13 and/or CD33 was found on the TdT^+ cells (Figure 1). However, in five leukemias these pan-myeloid markers were only expressed by < 20% of the TdT^+ cells. Additional testing for CDw65 in two of these five cases (patients M.L. and F.W.) revealed that a majority of the TdT^+ cells expressed CDw65. In one BM sample (patient A.H.) with 0.5% TdT^+ cells, it was found that 25% of the TdT^+ cells were $CD10^+$ and 70% of the TdT^+ cells were $CD33^+$. The $CD10^+$, TdT^+ cells were usually smaller and did express TdT stronger than the $CD33^+$, TdT^+ cells. The former probably represented a small population of precursor B cells, while the latter were assumed to be a part of the leukemia in this patient. In all other leukemic cell samples listed in Tables 3 and 4, no significant percentages of $CD10^+$, TdT^+ cells were demonstrated. In most TdT^+ AML, the CD34 antigen was expressed in stronger (i.e., a higher antigen density) as well as in higher percentages by TdT^+ cells than by TdT^- cells. In most leukemias expres-

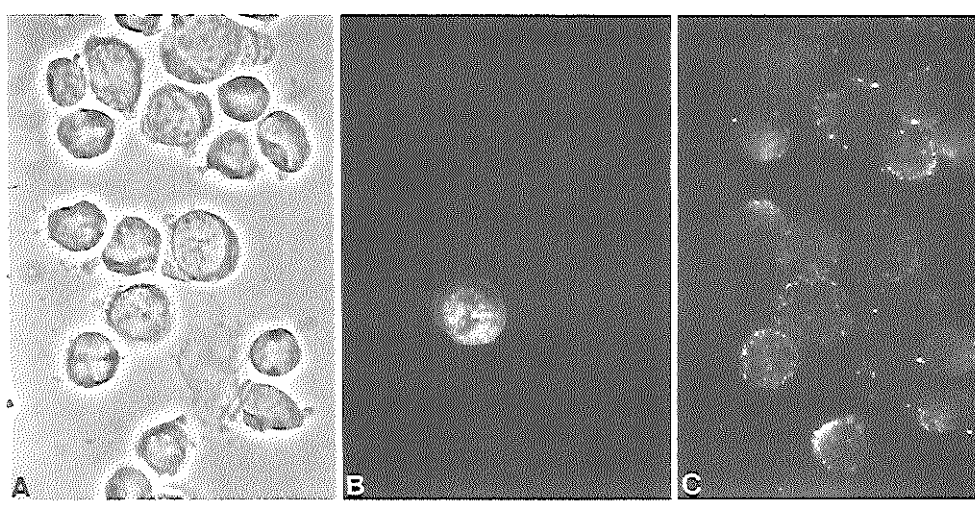

Figure 1. Double IF staining for CD33 and TdT on PB cells from patient P.B. at diagnosis. **A:** phase contrast morphology; **B:** TdT positive cell (FITC labeled); **C:** CD33 (My9) positive cells (TRITC labeled). The majority of the cells, including the TdT^+ cell, are positive for CD33.

TABLE 4. Twenty-one AML with >10% of myeloid marker$^+$, TdT$^+$ cells.

Patients	Age	Sex	WBC ($\times 10^9$/liter)	Blasts BM (%)	Blasts PB (%)	Hb (mmol/liter)	Platelets ($\times 10^9$/liter)	FAB diagnosis	Immunological marker analysis per MNC (per TdT$^+$ cells)[a]												
										TdT	CD2	CD3	CD7	CD10	CD19	CD13	CD14	CD15	CD33	CD34	HLA-DR
M.M.	17	M	1.7	71[b]	0	7.2	38	M6	BM	11	9(½)	10	10(5)	<1(½)	8(0)	2(39)	2(0)	38(0)	57(97)	5(26)	32(26)
N.A.	46	M	18	81	68	6.8	38	M2	PB	11	15(0)	13	13(0)	<1(1)	9(0)	5(1)	2(0)	1(6)	55(20)	10(87)	79(67)
B.B.	13	F	29	73	58	2.8	14	M4	BM	12	3	½	2(0)	0(0)		82(89)	3(0)	15(0)	92(95)		74(26)
F.H.	34	M	31	90	76	7.6	108	M5a	PB	12	43(0)	23	23(0)	0(0)	8(0)	25(68)	69(13)	0(0)	56(12)	36(88)	74(98)
A.V.	29	M	5.9	99	72	5.9	21	M2	BM	14	3(0)	½	20(8)	2(2)	3(1)	83(56)	0(0)	0(0)	87(65)	92(99)	91(70)
L.B.	60	M	110	70	34	6.3	35	M4	PB	15	29(0)	30	24(0)	0(0)	16(0)	14(1)	27(0)	3(17)	20(0)	31(88)	41(89)
A.S.	71	M	0.9	88	16	8.9	50	M1	BM	17	33(0)	26	23(0)	0(0)	0(0)	36(99)	½(0)	1(0)	14(54)	39(99)	3(0)
C.O.	86	F	8.0	87	81	5.9	15	M2	PB	17	10(0)	7	10(0)	0(0)	1(0)	85(98)	5(0)	0(0)	86(81)	85(93)	90(96)
P.A.	53	M	47	95	88	7.4	81	M3	BM	20	½(0)	2	½(0)	0		44(2)	1	0	98(97)	17(48)	½(2)
M.W.	70	F	3.2	80	29	5.5	115	M2	PB	22	23(½)	27	25(0)	<1(½)	5(0)	70(83)	28(0)	3(0)	35(71)	45(94)	6(2)
V.V.	76	M	3.7	71	12	5.4	50	M5b	BM	24	11	7	9	0	2	67(71)	50(16)	18(6)	29(0)	81(99)	
P.K.	16	F	21	39	18	3.6	29	M4	BM	25		5	2(3)	0(0)		72(85)	1	7	0(0)		85(97)
W.W.	45	M	3.6	50	1	9.0	153	M2[c]	BM	29	8	10	20(27)	1(1)		9(0)	3(0)	11(0)	55(99)		6
J.Z.	12	F	20	80	47	5.9	31	M2	BM	32	6(0)	5	7(0)	0(0)	2(0)	60(57)	2(0)	32(0)	89(99)	37(16)	11(17)
F.B.	53	F	56	95	82	4.6	17	M1	PB	45	20(0)	4	17(0)	0(0)	11(0)	89(85)	9	4	80(85)	83(99)	85(98)
F.W.	10	M	33	67	80	5.7	38	M2[d]	BM	47	3(0)	2	2(0)	0(0)	46(25)	45(11)[d]	3(0)	6(0)	55(6)[d]	43(97)	76(82)
C.A.	38	F	42	75	97	5.4	11	M1[e]	PB	61	½(0)	½	90(79)	0(0)	½(0)	92(87)	0(0)	0(0)	74(39)	78(63)	81(87)
B.M.	76	F	11	95	57	4.3	39	M0	BM	65	21(0)	7	13(½)	0(0)	21(0)	48(50)	9(0)	39(16)	80(90)	87(99)	87(94)
P.G.	63	F	30	83	95	5.4	261	M0	BM	69	11(0)	6	15(4)	0	½(0)	78(98)	6	2	16(1)		17(8)
H.B.	67	F	27	91	54	5.5	67	AUL	PB	78	22(0)	13	6(0)	0	3(0)	½(0)	0(0)	30(14)	½(0)	78(96)	83(90)
A.W.	44	F	26	96	89	4.4	38	M2	PB	83	8(0)	7	9(0)	0(0)	1(0)	39	1(0)	½(0)	97(99)	0(0)	3(0)

a. Percentages positivity for surface membrane marker per TdT positive cells as determined by double IF staining.
b. Including erytroblasts.
c. Analyses performed at first relapse.
d. CDw65 was found to be positive: 43% per MNC and 45% per TdT$^+$ cells.
e. Analyses performed at second relapse. In first relapse 1.5% CD13$^+$, TdT$^+$ cells were detected.

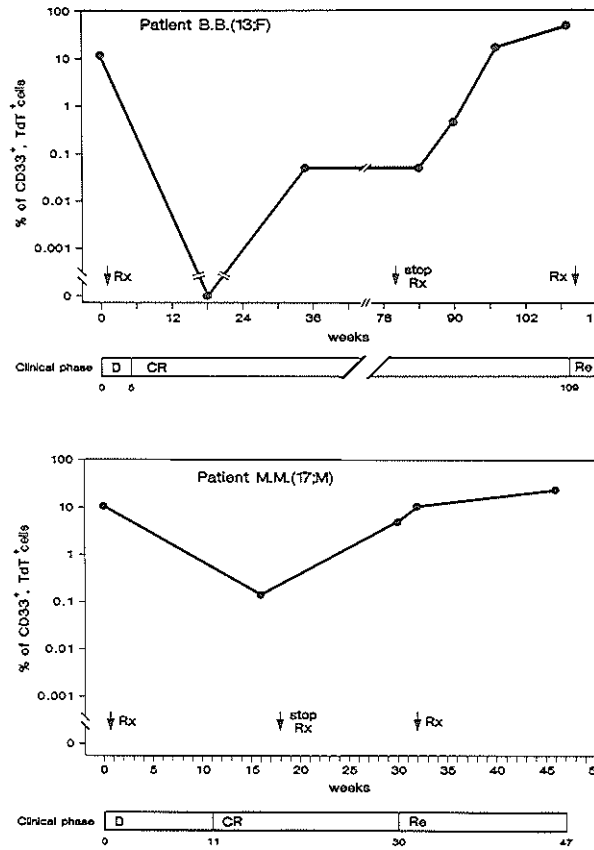

Figure 2. Follow-up of BM samples from two AML patients by use of double IF staining for TdT and the myeloid marker CD33(My9). Clinical phase is based on both clinical observation and cytomorphology of BM and PB samples. *D*, diagnostic phase; *CR*, complete remission; *Re*, relapse; *Rx*, start chemotherapy; *Stop Rx*, end of chemotherapy.

sion of HLA-DR by TdT^+ cells was comparable to the HLA-DR expression by the total population of MNC. However, especially in some leukemias classified as AML-M4 or AML-M5, the TdT^- cells expressed HLA-DR stronger and in higher frequencies than the TdT^+ cells. CD15 expression by TdT^+ cells was found in low percentages (< 20%) in 10 leukemias. Interestingly, two of these leukemias (patients B.M. and H.B.) were AML-M0 or AUL without any maturation into granulocytic cells according to morphologic criteria. $CD14^+$, TdT^+ cells were only demonstrated in two leukemias diagnosed as AML-M5a (patient F.H.) and AML-M5b (patient V.V.). CD19 expression by TdT^+ cells was found in three leukemias classified as AML-M2 (patients N.L., M.L., and F.W.). $CD2^+$, TdT^+ cells were demonstrated in three AML-M4 (patients A.K., M.B., and M.V.), while CD7 expression by > 20% of the TdT^+ cells was found in eight AML cases.

Follow-up of two AML patients

Follow-up studies were performed in patients B.B. and M.M. Hematologic data as well as

the immunological phenotypes of these two AML at diagnosis are given in Table 4. The results of the follow-up studies are summarized in Figure 2.

In both patients about 10% of the leukemic BM cells at diagnosis expressed both TdT and CD33. In patient B.B. 0.05% of $CD33^+$, TdT^+ cells were found in the BM 4 weeks after stopping therapy. Subsequently, the percentage of double positive cells gradually increased. Seven months after stopping therapy and 6 months after the first detection of $CD33^+$, TdT^+ cells a BM relapse occurred, as proven by cytomorphology. Patient M.M. achieved complete cytomorphologic remission 10 weeks after diagnosis. However, in a BM sample taken 6 weeks later, 0.14% $CD33^+$, TdT^+ cells were still present. Another 14 weeks later this patient relapsed.

DISCUSSION

According to the literature TdT expression can be found in 5%-46% of AML (7-16). The differences in incidence of TdT^+ AML can be explained by the types of AML examined as well as the sensitivity of the method used to detect TdT (9,11,13-15). In most publications an AML was regarded to be TdT^+ when at least 10% of MNC expressed TdT (10,11,13-15). In our study 35% (21 of 60) of the AML met this criterium (Table 4), a percentage which corresponds with some literature data (9,11-13). However, in eight out of these 21 leukemias, the percentage of TdT^+ cells was between 10% and 20%, and in only five leukemias did the majority (> 50%) of the AML cells express TdT. These results are in line with the observation that in most TdT^+ AML only a subpopulation of the AML cells is TdT^+ (7,9,12-14). This reflects the heterogeneity that is often seen in AML (19,20). Based on these observations we anticipated that also small TdT^+ subpopulations (<10% of MNC) may occur. Using double marker analysis for TdT and surface membrane markers, it was indeed found that in 40% of the AML in this study such a small TdT^+ subpopulation was present (Table 3). These TdT^+ cells generally expressed TdT weakly, had a myeloid appearance in phase contrast microscopy, expressed the same myeloid markers as the TdT^- leukemic cells, and were $CD10^-$. Therefore, it was concluded that these TdT^+ cells belonged to the leukemia. All together, in about 75% of AML a TdT^+ subpopulation taking up 0.1% to 83% of MNC was detected.

The occurrence of TdT^+ cells within an AML appears to be independent of the FAB diagnosis. Only within the "more mature" AML-M5 subgroup, TdT^+ cells were found in a minority of the leukemias (Table 1). This is in contrast with a report, which describes a higher incidence of TdT^+ AML among the monocytic variants (21). On the other hand, several investigators have associated TdT expression with immature myeloid leukemias (12,14,15,22,23). In this respect, the finding of a stronger (higher density) CD34 expression by TdT^+ cells as compared with TdT^- cells in most AML is interesting. CD34 is expressed by progenitor cells in normal BM as well as immature acute leukemias (24). Therefore, it is likely that the $CD34^+$, TdT^+ cells represent the immature subpopulation within an AML. Such an immature $CD34^+$, TdT^+ subpopulation was detected in the majority of AML but appeared to vary in size (Tables 3 and 4). The presence of a relationship between TdT expression and

immaturity is also suggested by a higher frequency of TdT$^+$ cells at relapse as compared to the frequency at diagnosis. This is illustrated by the data on the two patients in Figure 2 which document an expansion of the TdT$^+$ leukemic subpopulation from 10% at diagnosis to 48% (patient B.B.) or to 25% (patient M.M.) at relapse. Such an increase of the TdT$^+$ subpopulation was also observed in patient C.A. (Table 4), who had 61% TdT$^+$ cells at second relapse but only 1.5% CD13$^+$, TdT$^+$ cells at first relapse. The expansion of the TdT$^+$ subpopulation within an AML at relapse may reflect the reduced differentiation capacity of the leukemic cells (25). These combined data lead us to speculate that the TdT$^+$ leukemic subpopulation contains the AML cells with clonogenic properties.

We not only found a variation between the AML in the percentage of TdT$^+$ cells, but there was also a marked heterogeneity with respect to the surface membrane markers expressed by the TdT$^+$ cells (Tables 3 and 4). It is interesting to correlate the immunological phenotype of all MNC and especially of the TdT$^+$ cells with other features of the AML, such as cytomorphology and cytogenetics. We recently postulated a correlation between translocation (6;9) and the immunological phenotype HLA-DR$^+$, CD13$^+$, partly TdT$^+$ (26). However, such comparisons are often hampered by the morphologic and immunophenotypic heterogeneity present in most AML. This partly explains the poor correlation between immunophenotype and cytomorphologic diagnosis as reported in the literature (19,21,27-29) and as found in this study. These discrepancies can further be explained by the fact that Ficoll density separation for immunological marker analysis of MNC may enrich for more immature cells, while on the other hand the morphologic diagnosis is based on the presence of maturation characteristics, even when it concerns a minor leukemic subpopulation (e.g., 3% peroxidase or Sudan black positive cells for the diagnosis AML-M1) (17).

In our series of AML we did not find "mixed" acute leukemias, such as mixtures of relatively large populations of CD10$^+$, TdT$^+$ lymphoblasts and CD13$^+$, CD33$^+$ myeloblasts (30,31). However, in several AML a part of the leukemic cells expressed "lymphoid" markers such as CD2, CD7, or CD19. Whether this represents aberrant leukemia associated expression or whether normal counterparts of such cells occur can only be studied by careful double marker analysis of normal BM samples.

If TdT is expressed by the immature cells within the leukemia, it is interesting to monitor this immature subpopulation in AML patients during and after chemotherapy. This follow-up can be performed by use of double marker analysis for a particular myeloid marker and TdT. The choice of the myeloid markers should depend on immunophenotype of the leukemia at diagnosis. For the interpretation of the results of these double marker analyses, it is important to know whether each myeloid marker$^+$, TdT$^+$ cell can be regarded as malignant or whether such cells also occur in normal cell samples. Previous studies revealed that CD13$^+$, TdT$^+$ cells and/or CD33$^+$, TdT$^+$ cells are present in low frequencies (0.001% - 0.03%) in the majority of the BM samples from healthy volunteers and non-leukemia patients, while these cells are absent in most BM samples taken during and after chemotherapy from patients with an ALL in continuous complete remission (CR) (32,33). In PB samples from healthy volunteers and from patients with an ALL in continuous CR, such double positive cells are not detectable (32).

Therefore, a single finding of a low percentage of myeloid marker$^+$, TdT$^+$ cells (<0.03%) in BM should be interpreted with caution. When higher percentages (>0.03%) of myeloid marker$^+$, TdT$^+$ cells are detected, residual AML can be suspected, especially when the number of these cells increase during follow-up or when double positive cells are detected in PB. We recently started a follow-up study using double marker analysis for a myeloid marker and TdT in TdT$^+$ AML patients to detect minimal residual disease. Myeloid marker$^+$, TdT$^+$ cells could be demonstrated in BM samples from two patients in cytomorphologic CR who relapsed later on. As illustrated in Figure 2, a gradual increase of CD33$^+$, TdT$^+$ cells was observed in patient B.B. during the 6-months period before relapse occurred. Patient M.M., who was in CR for 20 weeks, never obtained remission based on the results of immunological marker analysis. Therefore, double marker analysis for a myeloid marker and TdT may be useful for the detection of minimal disease during follow-up of AML patients. Furthermore, such follow-up can provide information about responses upon different chemotherapeutical drugs as well as about growth kinetics of the tumor cells.

In conclusion, in the majority of AML, a TdT$^+$ subpopulation can be detected. These TdT$^+$ cells probably represent the immature leukemic subpopulation which may contain the clonogenic AML cells. Furthermore, our results indicate that double marker analysis for TdT and a myeloid marker may represent a powerful tool for detection of minimal residual disease as well as the detection of early relapse.

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CHAPTER 4.3

DETECTION OF RESIDUAL DISEASE IN AML PATIENTS BY USE OF DOUBLE IMMUNOLOGICAL MARKER ANALYSIS FOR TERMINAL DEOXYNUCLEOTIDYL TRANSFERASE AND MYELOID MARKERS*

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SUMMARY

In the majority of patients with acute myeloid leukemia (AML) immature leukemic subpopulations expressing myeloid markers and terminal deoxynucleotidyl transferase (TdT) are present. The normal counterparts of these double positive cells are rare in bone marrow (BM) (<0.03%; if they occur at all) and are not detectable in peripheral blood (PB). In 14 patients with TdT^+ AML at diagnosis we have performed a prospective follow-up study to monitor the myeloid $marker^+$, TdT^+ cells during and after chemotherapy.

One patient did not obtain complete remission (CR), a second patient relapsed under therapy, whereas the other 12 patients were in cytomorphological CR at the end of chemotherapy. During subsequent follow-up seven of these 12 patients developed one or two relapses (total of ten relapses). Nine of these ten relapses were preceded by a gradual increase of myeloid $marker^+$, TdT^+ cells in BM and PB samples over a period of (14-38 weeks). In one patient the relapse was not preceded by a gradual increase of double positive cells. This false negative result was caused by a phenotypic shift, since at relapse the AML cells did not express TdT.

In the five AML patients who still are in continuous cytomorphological CR for 32-46 months we repeatedly detected relatively high percentages of myeloid $marker^+$, TdT^+ cells in BM (up to 0.1%) and PB (up to 0.02%). Although we could not prove the leukemic origin of these double positive cells, they might represent residual dysplastic AML cells which survived chemotherapy but which are not capable of causing leukemia regrowth as yet. This would be in line with recent polymerase chain reaction (PCR) studies, which could demonstrate the

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persistence of leukemic clones in the majority of AML patients in continuous CR.

It is concluded that double immunofluorescence (IF) labeling for myeloid markers and TdT is a powerful tool to detect residual disease in TdT^+ AML patients. A gradual increase of double positive cells is highly suggestive for leukemic cell regrowth and can be used to predict relapse.

INTRODUCTION

During the last decades progress has been made in the treatment of AML. Intensive chemotherapy results in a remission rates of 60%-80%, but without bone marrow transplantation (BMT) the majority of patients relapse (1-3). Depending on their age, only 15% to 40% of AML patients are curred, i.e. achieve 5-year disease for survival (1-3). Cure rates of 40%-50% have been reported after allogeneic BMT (4,5) or after a combination of high dose chemotherapy and autologous BMT (ABMT) (6). Apparently, low numbers of leukemic cells persist, in most AML patients although they are undetectable by conventional cytomorphological techniques. These methods have a detection limit of 1% to 5% (1 to 5 leukemic cells between 100 normal cells), which implies that over 10^{10} AML cells might remain undetectable (7-9). Therefore, more sensitive techniques are needed to evaluate the effectiveness of the applied treatment, i.e. the reduction of tumor mass. Such information is essential for adaptation of treatment protocols. Sensitive techniques can also be helpful to recognize the development of relapse in an early phase and they can be used to determine the optimal time point for harvesting BM grafts for ABMT and to screen the obtained autologous grafts for the presence of residual leukemic cells.

Several methods for the detection of minimal residual disease (MRD) in AML have been evaluated, including morphology, cytogenetics (10-14), cell culture systems (15,16), cell kinetic analysis (17), immunological marker analysis (8,18,19), molecular biological techniques (20-28), cancer procoagulant assay (29), and magnetic resonance imaging (30-32). The application of cytogenetics or molecular biological techniques requires the presence of a clonal chromosome aberration, a gene rearrangement, or a mutation. If the aberration is known precisely, the PCR technique can theoretically detect tumor specific sequences with a detection limit down to 10^{-5} (1 leukemic cell between 10^5 normal cells) (9,25,26). At present, most methods for MRD detection are only applicable in a minority of AML patients. The PCR technique can be applied in AML patients with a specific well-defined chromosome aberration, such as t(9;22), t(15;17), or t(6;9), or with cross-lineage rearrangements of immunoglobulin (Ig) and/or T cell receptor (TcR) genes (9,24-28,33-36). Cytogenetics and cell culture systems are only useful in AML which proliferate *in vitro*. An additional problem in the application of most methods is that quantification of the tumor burden is difficult or impossible.

The use of immunological marker analysis for detection of MRD is hampered by the fact that the immunophenotype of AML cells is comparable to that of normal immature hematopoetic cells (37,38). This implies that the presence of these normal cells limits the immunological detectability of the leukemic cells. For most markers this results in detection limits, which are

not lower than 1% to 10% (10^{-2} to 10^{-1}) (8,18). Using double IF staining for TdT and the pan-myeloid markers CD13, CD33, or CDw65, we could demonstrate expression of TdT in ~75% of AML (39). In contrast to acute lymphoblastic leukemias (ALL), in AML the TdT expression is often restricted to a subpopulation of the AML cells, which is illustrated by the finding that in half of the TdT$^+$ AML the frequency of myeloid marker$^+$, TdT$^+$ cells is less than 10%. Interestingly, the precursor antigen CD34 is expressed in much higher frequencies on the TdT$^+$ leukemic cells than on the TdT$^-$ leukemic cells, suggesting that the TdT$^+$ cell fraction represents an immature subpopulation, which might contain the clonogenic AML cells (39). Extensive control studies have revealed that normal counterparts of the myeloid marker$^+$, TdT$^+$ cells are rare in BM (<0.03%, if they occur at all) and that such cells are not detectable in PB (40,41). Using fluorescence microscopy, TdT$^+$ cells are reliably detectable on cytocentrifuge preparations with a detection limit of 0.01% to 0.001% (10^{-4} to 10^{-5}), depending on the number of cells scanned (42,43).

To investigate whether double IF staining for a myeloid marker and TdT can be used to detect MRD in AML patients we performed a prospective follow-up study to monitor myeloid marker$^+$, TdT$^+$ cells in patients with a TdT$^+$ AML during and after therapy. Fluorescence microscopy was used to identify the myeloid marker$^+$, TdT$^+$ cells. Although double IF staining for TdT and surface membrane markers can be performed by use of flow cytometry, the detection limit of this method is not as low as the microscopic method (44-46). Furthermore, microscopy enables the identification of the typical nuclear expression pattern of TdT as well as some morphological aspects at the single cell level, whereas flow cytometry can not identify subcellular staining patterns and only allows reliable analysis of cell populations instead of individual cells.

MATERIALS AND METHODS

Patients

Fourteen patients (aged 3-73 years) were diagnosed to have AML. The diagnosis was based on the cytomorphology of PB and BM smears stained for May Grünwald Giemsa and cytochemistry (Sudan black B, myeloperoxidase, periodic acid-schiff, and α-naphtylacetate esterase). The patients were classified according to the revised French American British (FAB) criteria (47). Treatment was instituted according to the ANLL-87 protocol of the Dutch Childhood Leukemia Study Group (DCLSG) in case of children (<16 years). Adults were treated according to the AML-4 protocol (16-60 years of age) of Stichting Hemato-Oncologie voor Volwassenen Nederland (HOVON) or AML-11 protocol (>60 years of age) of the EORTC leukemia group and HOVON. All three chemotherapy regimens are outlined in Table 1. According to the treatment protocols patients were examined frequently. At regular intervals PB and BM samples were evaluated for cellularity and cytomorphology. CR was defined by less than 5% blasts in a normocellular BM with normal PB cell counts including more than 1×10^9/l granulocytes and more than 100×10^9/l platelets and the absence of extramedullary leukemic manifestations. Partial remission (PR) was defined as a BM showing more than 5% but fewer than 25% blasts and PB showing more than 0.5×10^9/l granulocytes and more than 25×10^9/l platelets.

TABLE 1. Treatment protocols.

DCLSG-ANLL-87 protocol			
Induction treatment	Ara-C	100 mg/m^2	14x
	DNR	30 mg/m^2	6x
	Etoposide	150 mg/m^2	3x
Consolidation treatment consisting of Prednison, 6 Thioguanine, Vincristine, Adriamycin, Ara-C, and Cyclophosphamide			
Intensification treatment (2 courses)	HD-Ara-C	3000 mg/m^2	6x
	Etoposide	125 mg/m^2	4x
HOVON-AML-4 protocol			
Course I	Ara-C	200 mg/m^2	7x
	DNR	45 mg/m^2	3x
Course II	Ara-C	2000 mg/m^2	6x
	m-AMSA	120 mg/m^2	3x
Course III	Mitoxantrone	10 mg/m^2	5x
	Etoposide	100 mg/m^2	5x
Courses at 4 weeks intervals			
EORTC-HOVON-AML-11 protocol			
Randomization to either standard therapy or standard therapy plus GM-CSF (5 μg/kg 28x or until granulocytes reach 0.5x10^9/l)			
Induction treatment	Ara-C	200 mg/m^2	7x
	DNR	30 mg/m^2	3x
In case of CR one course, in case of PR two identical courses			
Consolidation treatment	Ara-C	200 mg/m^2	7x
	DNR	30 mg/m^2	1x
Complete responders are randomized to no further treatment or maintenance treatment of Ara-C (20 mg/m^2 8x)			

Abbreviations used: Ara-C = cytosine arabinoside, DNR = daunorubicin, m-AMSA = amsacrine.

Immunological marker analysis

Cell samples.

Mononuclear cells (MNC) were isolated from BM and PB samples by Ficoll Paque (density 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. Immunological marker analysis was performed on freshly isolated MNC.

At diagnosis.

The MNC from BM and/or PB were incubated with monoclonal antibodies (McAb) as described (48). Several McAb were used, including the B cell markers CD19 (B4; Coulter Clone, Hialeah, FL, USA) and CD10 (VIL-A1; Dr. W. Knapp, Vienna, Austria); the T cell markers CD2 (T11; Coulter Clone), CD3 (Leu-4; Becton Dickinson, San Jose, CA, USA), and CD7 (3A1; American Type Culture Collection, Rockville, MD, USA); the myeloid monocytic markers

CD13 (My7; Coulter Clone), CD14 (My4; Coulter Clone), CD15 (VIM-D5; Dr. W. Knapp), CD33 (My9; Coulter Clone), and CDw65 (VIM-2; Dr. W. Knapp); the erythroid marker glycophorin (Gp) A (VIE-G4; Dr. W. Knapp); the platelet markers CD42a (FMC25; Seralab, Crawley Down, UK) and CD61 (C17; Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands) and the non-lineage specific markers CD34 (BI-3C5; Seralab) and HLA-DR (L243; Becton Dickinson). Surface membrane markers were visualized by use of a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service) and evaluated with a FACScan flow cytometer (Becton Dickinson). A leukemia was regarded to be positive for a surface membrane marker if more than 20% of the MNC expressed the marker. The expression of TdT was detected as described by use of a rabbit anti-TdT antiserum and a FITC-conjugated goat anti-rabbit Ig antiserum (Supertechs, Bethesda, MD, USA) (48). Double IF staining for TdT and several surface membrane antigens, i.e. CD2, CD7, CD10, CD13, CD14, CD15, CD19, CD33, CD34, CDw65, and a mixture of CD13 and CD33 were performed as described previously (39,42,48). The binding of the McAb on the surface membrane was demonstrated by use of a tetramethylrhodamine isothiocyanate (TRITC) conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service). The TdT IF labelings were analyzed on Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany), equipped with phase contrast facilities (48). The percentages of TdT$^+$ cells were determined by analyzing at least 1000 MNC. In case of double IF staining at least 200 TdT$^+$ cells were analyzed if present; when MNC samples contained less than 1% of TdT$^+$ cells, two cytocentrifuge preparations (total ~50,000 MNC) were scanned per double IF staining. In the latter cases the total number of MNC was calculated from the number of MNC found in three different microscopic fields, which were representative for the whole cytocentrifuge slide.

During Follow-up.

If myeloid marker$^+$, TdT$^+$ cells were detected at diagnosis, the patient was included in the follow-up study. BM and PB samples, regularly taken for cytomorphological evaluation, were investigated for the presence of myeloid marker$^+$, TdT$^+$ cells. For detection of myeloid marker epression we used a mixture of CD13 and CD33 McAb. In one case (patient P.Z.), we also analyzed the BM and PB samples for the presence of CDw65$^+$, TdT$^+$ cells. Labeling as well as analysis were performed as described above, which implies that at least either 200 TdT$^+$ cells or all TdT$^+$ cells in two cytocentrifuge preparations were analyzed. The results of this immunological follow-up were not used to adapt the treatment modality.

RESULTS

Patients

Age, sex, white blood cell (WBC) count, percentage of blast cells in BM and PB, and FAB diagnosis of the 14 AML patients are given in Table 2. The five patients who were <16 years of age were treated according to the DCLSG-ANLL-87 protocol, seven adult patients were treated according to HOVON-AML-4, and two adult patients according to EORTC-HOVON AML-11 (Tables 1 and 2). Follow-up data concerning the clinical phase and the treatment of each individual patient are given in Figures 1-4.

Thirteen patients obtained CR within 3-41 weeks after start of treatment. One patient (V.V.) did not obtain remission and died 18 weeks after AML diagnosis (Figure 1). Patient J.V. relapsed under chemotherapy 24 weeks after diagnosis (Figure 1). Seven patients, who were in CR at the end of chemotherapy, developed one or two relapses (total of ten relapses) 7-27 months after diagnosis (Figures 2 and 3). The other five patients are in first CR for 32-46

TABLE 2. Characterization of the 14 AML at diagnosis.

Patients	Age	Sex	WBC ($\times 10^9$/liter)	Blasts BM (%)	Blasts PB (%)	FAB diagnosis	Immunological marker analysis per MNC (per TdT$^+$ cells)[a]										
								TdT	CD3	CD10	CD13	CD14	CD15	CD33	CD34	CDw65	HLA-DR	Treatment protocol
V.V.	43	F	33	70	65	M1	BM	1.2	9	3(12)	70(54)	14	4	77(81)	45(98)	41(35)	67	HOVON-AML-4
J.V.	66	F	28	79	81	M2	BM	41	5	<1(0)	83(46)	1	6	92(98)	4(3)	28(0)	17	HOVON-AML-11
A.K.	26	M	82	90	75	M4	BM	1.8	3	2(0)	95(75)	29	4	76(25)	93(99)	69(91)	67	HOVON-AML-4
M.V.	13	F	6.5	57	31	M4	BM	7	8	0(0)	81(69)	38	0	76(45)	55(81)	56(45)	60	DCLSG-ANLL-87
J.Z.	12	F	20	80	47	M2	BM	32	5	0(0)	60(57)	2	32	89(99)	37(22)	56(11)	11	DCLSG-ANLL-87
P.Z.	3	M	9.5	73	36	M2	BM	25	9	0(0)	82(9)	10	72	73(50)	24(80)	82(79)		DCLSG-ANLL-87
R.G.	24	M	4.2	80	65	M2	BM	20	13	<1	38(21)	1	8	59(87)	66(90)	15	70	HOVON-AML-4
R.R.	3	M	21	98	85	M1	BM	0.1	<1	<1(25)	68(3)	0	21	96(69)	<1(39)	68	1	DCLSG-ANLL-87
B.S.	73	F	43	99	86	M1	BM	1.7	<1	1(3)	<1(0)	<1	<1	88(99)	<1(½)	2(2)	2	HOVON-AML-11
M.K.	58	F	180	86	96	M2	PB	0.3	3	0(0)	2(0)	8	<1	89(74)	<1(0)	15(82)	10	HOVON-AML-4
N.A.	46	M	18	81	68	M2	PB	11	13	<1(1)	45(20)	2	37	55(20)	70(87)	25(14)	79	HOVON-AML-4
K.B.[b]	9	F	9.5	65	59	M1	BM		4	2	0	5	16	58	5	21	71	DCLSG-ANLL-87
J.B.	45	M	11	92	3	M5b	PB	0.03	5	<1(0)	32(77)	87	8	95(72)	86(0)	87(0)	90	HOVON-AML-4
C.K.	34	F	70	96	80	M2	PB	0.03	4	0	59	1	8	91(67)	3(99)	21	42	HOVON-AML-4

a. Percentage positivity for surface membrane marker per TdT positive cells as determined by double IF staining.
b. In this patient analysis for TdT as well as double IF labelings for myeloid markers and TdT were performed 4 weeks after start of treatment (see text).

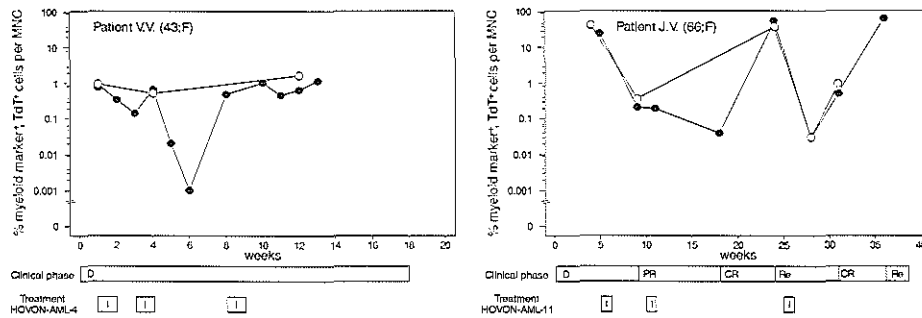

Figure 1. Follow-up of two AML patients, who did not obtain CR (patient V.V.) or relapsed under chemotherapy (patient J.V.), by use of double IF staining for TdT and a myeloid marker [CD13 (My7) or CD33 (My9)]. Clinical phase is based on both clinical observations and cytomorphology of BM and PB samples. Symbols: ●–●, PB samples; ○–○, BM samples. Abbreviations: *D*, diagnosis; *CR*, complete remission; *Re*, relapse; *I*, induction treatment; *C*, consolidation treatment; *MNC*, mononuclear cells.

months (Figure 4).

To perform ABMT, BM from five patients was harvested 1-19 weeks after obtaining CR. They received an ABMT 8-29 weeks after CR. Two patients (A.K. and R.G.) subsequently relapsed (Figure 2), while the other three patients (M.K., J.B., and C.K.) are in first CR (Figure 4).

Immunological marker analysis

At diagnosis.

Table 2 the results of the analyses for TdT, CD3, CD10, CD13, CD14, CD15, CD33, CD34, CDw65, and HLA-DR as well as the expression of the various differentiation markers on the TdT$^+$ cells are given. In patient K.B. analysis for TdT expression as well as double IF stainings for myeloid markers and TdT were not performed at diagnosis, but 4 weeks after start of treatment.

In all cases the AML cells were CD33$^+$ and in 11 patients also CD13$^+$. Positivity for the pan-myeloid marker CDw65 was also found in 11 AML (Table 2). The three AML classified as either AML-M4 or AML-M5b were CD14$^+$. Three AML-M2 and one AML-M1 expressed CD15. All but five AML were HLA-DR$^+$. The AML were Gp A$^-$ and CD42a$^-$. In one AML (R.G.), 44% of CD61$^+$ cells were detected while other platelet markers (e.g. CD42a and CD62) were negative. In all other AML platelet markers were found to be negative. Expression of lymphoid differentiation markers was demonstrated in six AML. This concerned weak positivity of CD19 in patients P.Z. and N.A., positivity for CD2 in patients A.K. and M.V., positivity for CD7 in patient J.B., and expression of both CD2 and CD7 in patients V.V. and R.G. Eight AML were CD34$^+$, three additional AML contained 3%-10% CD34$^+$ cells, and in the other three AML $<1\%$ CD34$^+$ were detected (Table 2).

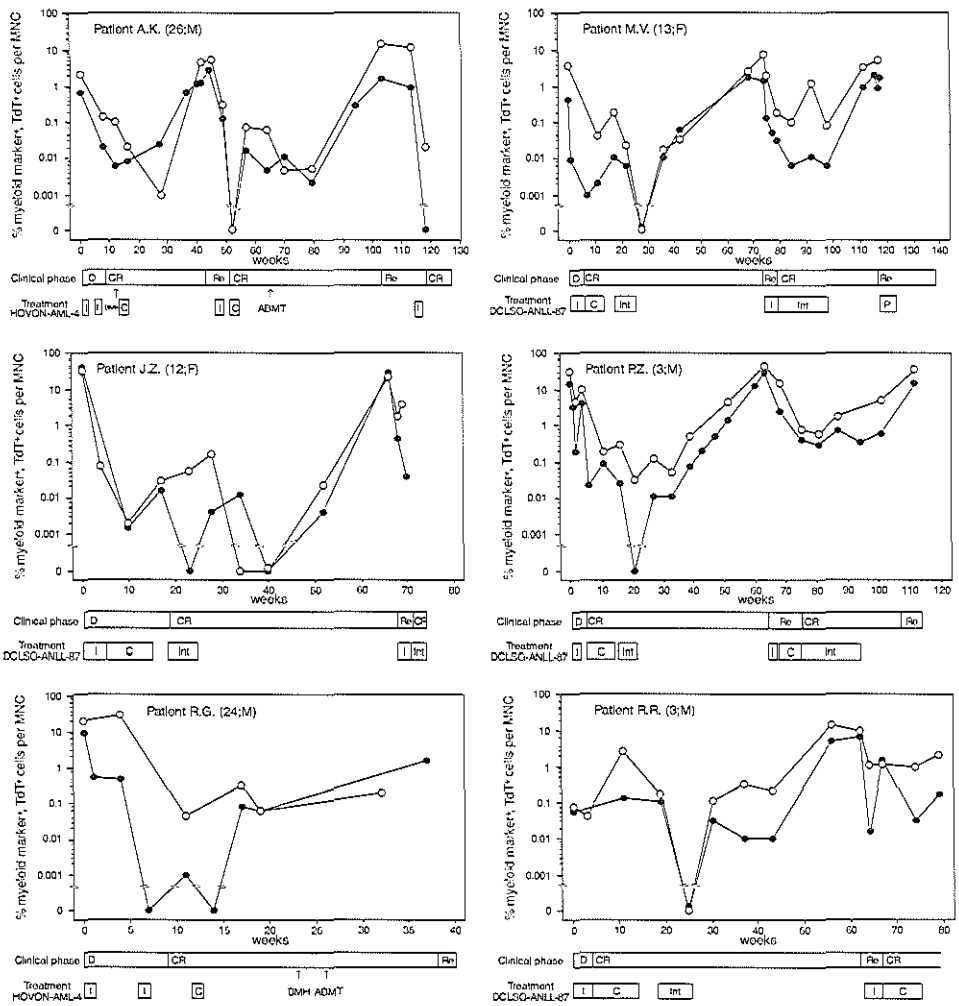

Figure 2. Follow-up of six AML patients, who relapsed after termination of chemotherapy, by use of double IF staining for TdT and a myeloid marker [CD13 (My7) or CD33 (My9)]. Clinical phase is based on both clinical observations and cytomorphology of BM and PB samples. Symbols: ●–●, PB samples; ○–○, BM samples. Abbreviations: *D*, diagnosis; *CR*, complete remission; *Re*, relapse; *I*, induction treatment; *C*, consolidation treatment; *Int*, intensification treatment; *P*, palliative treatment; *BMH*, bone marrow harvest; *ABMT*, autologous bone marrow transplantation; *MNC*, mononuclear cells.

In the 13 patients investigated at diagnosis the percentage of TdT^+ cells varied from 0.03% to 32% (Table 2). By use of double IF staining we could demonstrate that in 12 AML the majority of TdT^+ cells expressed the myeloid markers CD13, CD33, and/or CDw65 (Table 2). In patient N.A. only 20% of the TdT^+ cells was myeloid $marker^+$. The TdT^+ cells were $CD34^+$ in nine AML while in the other four AML <5% of the TdT^+ cells expressed CD34 (Table 2). In each AML sample the percentage of $CD10^+$, TdT^+ cells was <1% (Table 2). The TdT^+ cells

were negative for CD14 and CD15. Expression of CD2 was found on the TdT$^+$ cells in two patients (A.K., M.V.), CD7 in two other patients (V.V., R.G.), and CD19 in patients P.Z. and N.A.

In patient K.B. analysis for TdT expression was performed 4 weeks after start of chemotherapy. At that time BM and PB contained 1.1% and 0.11% of TdT$^+$ cells, respectively. Double IF staining revealed that 22% of the TdT$^+$ cells in BM and 4% of the TdT$^+$ cells in PB expressed CD33.

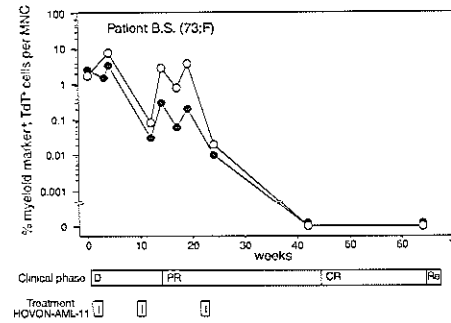

Figure 3. Follow-up of patient BS, by use of double IF staining for TdT and a myeloid marker [CD13 (My7) or CD33 (My9)]. Clinical phase is based on both clinical observations and cytomorphology of BM and PB samples. Symbols: ●–●, PB samples; ○–○, BM samples. Abbreviations: *D*, diagnosis; *PR*, partial remission; *CR*, complete remission; *Re*, relapse; *I*, induction treatment; *MNC*, mononuclear cells.

During Follow-up.

We monitored the percentage of (CD13 and/or CD33)$^+$, TdT$^+$ cells in all 14 patients during and after treatment. The results of this follow-up study are given in Figures 1-4.

PB from patient V.V., who did not obtain remission, was monitored weekly. The percentage of myeloid marker$^+$, TdT$^+$ cells only slightly decreased during and after the first chemotherapy course. Immediately after the second chemotherapy course the percentage of myeloid marker$^+$, TdT$^+$ cells declined, followed by a rapid increase to pre-treatment levels within 2 weeks. The third chemotherapy course seemed not to effect the myeloid marker$^+$, TdT$^+$ cell population (Figure 1). In patient J.V., who relapsed under chemotherapy, the number of myeloid marker$^+$, TdT$^+$ cells declined under chemotherapy but double positive cells remained detectable. At cytomorphological relapse the percentages of myeloid marker$^+$, TdT$^+$ cells were comparable with the percentages found at diagnosis (Figure 1).

Figures 2 and 3 give the follow-up results of the seven patients who relapsed after termination of chemotherapy. Three patients (A.K., M.V., P.Z.) developed two relapses. During treatment the percentages of myeloid marker$^+$, TdT$^+$ cells decreased, but low frequencies of double positive cells remained detectable after cytomorphological CR was obtained. In the four children of this group, who were treated according to the DCLSG-ANLL-87 protocol, the percentages of myeloid marker$^+$, TdT$^+$ cells decreased upon induction treatment, but increased during subsequent consolidation treatment. Eventually the percentages of myeloid marker$^+$, TdT$^+$ cells decreased sharply after two intensification courses with high doses Ara-C (Figure 2).

In six of the seven relapse patients we observed a gradual increase of myeloid marker$^+$, TdT$^+$ cells over a period of 14-38 weeks before cytomorphological relapse(s) (total of nine relapses) (Figure 2). In six of the eight relapses analyzed, the percentage of myeloid marker$^+$, TdT$^+$ was higher than the percentages found at initial diagnosis or at previous relapse. In patient P.Z. the percentages of CDw65$^+$, TdT$^+$ cells paralleled the percentages of (CD13

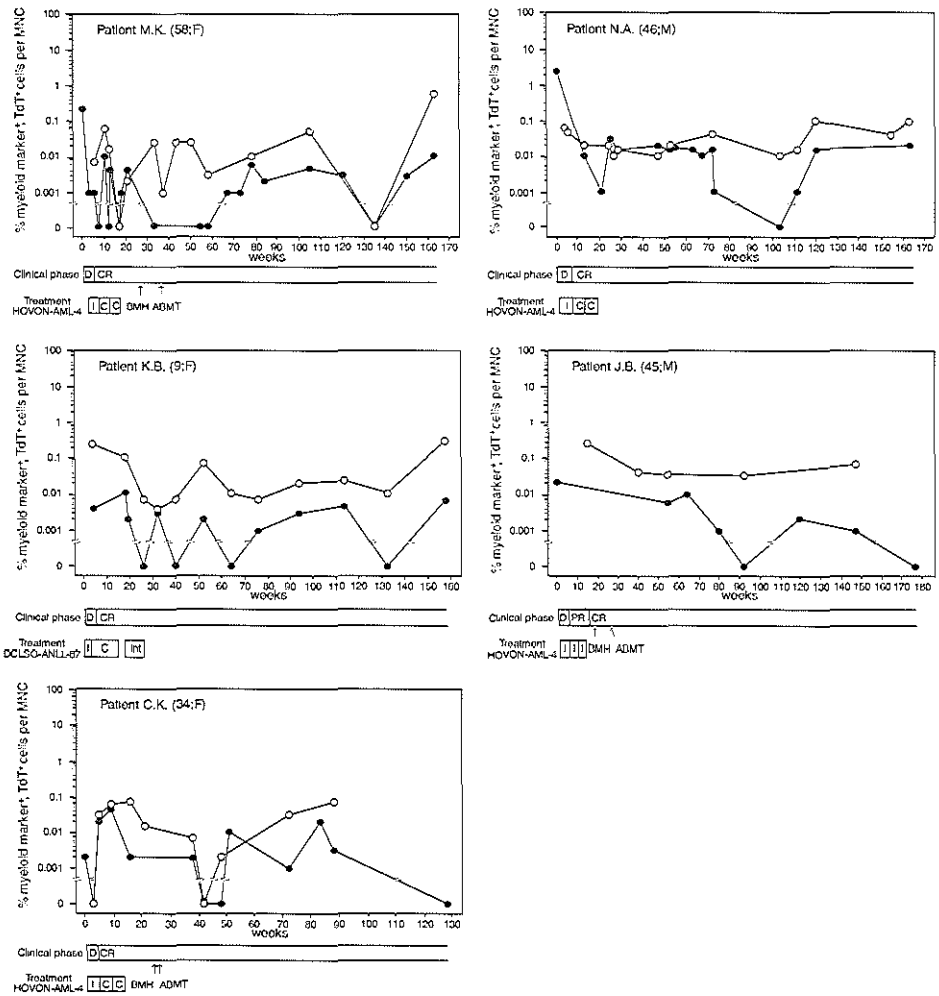

Figure 4. Follow-up of five AML patients, who are in first continuous CR, by use of double IF staining for TdT and a myeloid marker [CD13 (My7) or CD33 (My9)]. Clinical phase is based on both clinical observations and cytomorphology of BM and PB samples. Symbols: ●–●, PB samples; ○–○, BM samples. Abbreviations: *D*, diagnosis; *CR*, complete remission; *PR*, partial remission; *I*, induction treatment; *C*, consolidation treatment; *BMH*, bone marrow harvest; *ABMT*, autologous bone marrow transplantation; *MNC*, mononuclear cells.

and/or CD33)$^+$, TdT$^+$ cells. Two patients (R.G. and A.K.) received an ABMT during first cytomorphological CR (R.G.) or second cytomorphological CR (A.K.). The autologous BM transplant of patient A.K., which was taken during first CR, was found to contain 0.1% of myeloid marker$^+$, TdT$^+$ cells.

In patient B.S. a decrement of the myeloid marker$^+$, TdT$^+$ subpopulation was observed after the third chemotherapy course when the patient obtained CR (Figure 3). At relapse the AML cells were TdT$^-$. This explains the lack of double positive cells during the period before

relapse occurred.

In Figure 4 the follow-up results of the five patients in continuing first CR are given. After termination of chemotherapy low frequencies of myeloid marker$^+$, TdT$^+$ cells were repeatedly detected in BM (<0.001%-0.1%) and/or PB (<0.001%-0.02%). Three patients (M.K., J.B., C.K.) received an ABMT. The transplant of patient J.B. was analyzed and was found to contain 0.1% of myeloid marker$^+$, TdT$^+$ cells.

DISCUSSION

We prospectively investigated the value of double IF stainings for myeloid markers and TdT for detection of MRD in 14 patients with TdT$^+$ AML at diagnosis. During follow-up, one patient did not obtain remission and a second patient relapsed under chemotherapy, while the other 12 patients were still in cytomorphological CR at the end of chemotherapy. During subsequent follow-up a total of ten relapses occurred in seven patients. Nine out of these ten relapses were preceded by a gradual increase of myeloid marker$^+$, TdT$^+$ cells over a period of 14-38 weeks (median 31 weeks), which strongly suggests that it is possible to monitor leukemic cell growth and to predict a cytomorphological relapse (Figure 2). Interestingly, the percentages of myeloid marker$^+$, TdT$^+$ cells in PB paralleled those in BM, indicating that AML patients can probably be monitored by frequent analysis of their PB only. A comparable phenomenon has been found during follow-up of T-ALL patients using double IF stainings for a T cell marker and TdT (9,42). The relapse in one patient (B.S.) was not detected earlier than by use of conventional cytomorphology, because the leukemic cells of this patient at relapse were TdT$^-$ (Figure 3). Although loss of antigen expression or lineage switching has not been reported frequently in AML (8,49,50), this case demonstrates that such a phenomenon might be responsible for false negative results in the detection of MRD. Comparable to previous results, in almost all relapsed patients we found a relative increase of the myeloid marker$^+$, TdT$^+$ subpopulation at relapse when compared to the relative size of this population at diagnosis (39). The expansion of the TdT$^+$ AML subpopulation and their CD34 positivity in most patients supports the hypothesis that TdT$^+$ leukemic cells represent an immature leukemic subpopulation and provides additional proof that it is relevant to monitor this subpopulation for the detection of MRD (39,51).

Myeloid marker$^+$, TdT$^+$ cells were not only demonstrated in the patients who eventually relapsed, but low frequencies of double positive cells were also detected in BM (up to 0.1%) and PB (up to 0.02%) of the five patients who still are in first continuous cytomorphological CR for 32-46 months (Figure 4). The myeloid marker$^+$, TdT$^+$ phenotype is not leukemia-specific, since such cells have been found in BM samples of healthy volunteers and non-leukemia patients (40,41). However, myeloid marker$^+$, TdT$^+$ cells in normal BM are rare (<0.03%, if they occur at all) and such double positive cells are not detectable in normal PB. Myeloid marker$^+$, TdT$^+$ cells seem to occur more frequently in BM at older ages (40,41) and we have demonstrated a relatively high proportion of myeloid marker$^+$, TdT$^+$ cells in the BM and PB

of patients with myelodysplastic syndromes (unpublished results). Given the relatively large myeloid marker$^+$, TdT$^+$ cell population in several of the AML patients in CR, it could be argued that the cytostatic treatment induced expansion of the normal myeloid$^+$/TdT$^+$ cells. However, in most ALL patients in CR during and after chemotherapy no myeloid marker$^+$, TdT$^+$ cells were found (41). Therefore, we assume that the increased frequency of myeloid marker$^+$, TdT$^+$ cells in AML patients during first continuing CR is caused by residual dysplastic AML cells, which survived chemotherapy but which are not capable of causing leukemia regrowth as yet. This assumption is supported by data from the literature: A, Studies in AML patients with polymorphic X-chromosome-linked enzymes demonstrated so-called clonal remission in ~30% of cases (52,53). B, *In vitro* culture in combination with immunological marker analysis demonstrated the persistence of leukemic clones in the majority of AML patients in CR (15,54). C, Preliminary PCR studies on AML-M3 patients with t(15;17) show that leukemia-specific PML/RAR-α transcripts can be identified in the majority of patients during the first year of CR (26-28). D, Late relapses can occur more than 5 years after diagnosis in AML patients (55-57).

To determine whether the residual myeloid marker$^+$, TdT$^+$ cells in AML patients during first continuing CR are indeed leukemic, the PCR technique and the double IF staining technique have to be used next to each other. However in our group of 14 patients the PCR technique could not be used for MRD detection, since no suitable chromosome aberration was present, nor could we demonstrate clonal Ig or TcR gene rearrangements (34). Therefore, long-term follow-up of our patients should reveal whether our assumption is correct.

The sensitive double IF staining method for the detection of low numbers of AML cells is not only useful for the early detection of a relapse, but it can also give more insight in the biology of AML under treatment. The method can be applied to determine the sensitivity for the TdT$^+$ AML cells to the different courses of cytostatics and the results might be used for adaptation or stratification of treatment protocols. After start of treatment we observed a decline of the myeloid marker$^+$, TdT$^+$ cell population in most patients. In patient V.V., who did not obtain remission, the results suggested that the TdT$^+$ AML cells were only sensitive for a combination of Ara-C and m-AMSA, but not for the other chemotherapy courses (Figure 1). In the children who were treated according to the DCLSG-ANLL-87 protocol, an increase of the myeloid marker$^+$, TdT$^+$ cell population was seen during consolidation treatment, followed by a rapid decrease during intensification treatment (Figures 2 and 4). This observation suggests that the TdT$^+$ leukemic cells are not sensitive for the cytostatic regimen during consolidation treatment.

Although not the aim of this study, our data suggest that frequent analysis performed at regular intervals may be used to determine the growth rate of AML cells during relapse (58). The first relapse of patient P.Z. was preceded by a gradual increase of myeloid marker$^+$, TdT$^+$ cells in the PB with a 3-fold increase every 4 weeks (Figure 2). During this period both WBC count and WBC differentiation stayed relatively constant. Therefore, the doubling time of the AML population during relapse in this patient was estimated to be 17 days. This is essentially slower than the doubling time of 6.5 days, which was found in a T-ALL patient during relapse (58). In a recently published case of inadvertent transmission of AML from an affected donor

to a chronic myeloid leukemia patient in CR, the recipient developed AML 6 months after she received 1.2 x 10^8 myeloid blast cells per kilogram body weight (59). Assuming that this AML grew exponentially, a doubling time of ~15 days can be deduced, which is in line with our results.

In conclusion, our results demonstrate that double IF staining for myeloid markers and TdT represents a powerful tool for the detection of MRD in patients with TdT$^+$ AML. Low frequencies of myeloid marker$^+$, TdT$^+$ cells (<0.1% in BM and <0.02% in PB) should be interpreted with caution, while a gradual increase of double positive cells is highly predictive for an imminent relapse.

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CHAPTER 4.4

IMMUNOGLOBULIN AND T CELL RECEPTOR GENE REARRANGEMENTS IN ACUTE MYELOID LEUKEMIAS

Analysis of 54 cases and a review of the literature*

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SUMMARY

Fifty-four unselected acute myeloid leukemias (AML) were analyzed for their immunophenotype, especially the expression of terminal deoxynucleotidyl transferase (TdT), as well as for rearrangements and/or deletions in the immunoglobulin (Ig) heavy (IgH), Igκ, Igλ, T cell receptor (TcR)-β, TcR-γ, and TcR-δ/α genes. In 15% (8/54) of the AML patients one or more genes were rearranged. This especially concerned IgH gene rearrangements (seven cases) and to a lesser extent rearrangements of Igκ genes (one case), TcR-β genes (three cases), TcR-γ genes (two cases), and TcR-δ genes (two cases). Combined results from this study and from literature data on 378 unselected AML revealed that IgH gene rearrangements occurred in 14% of AML and Igκ gene rearrangements in 2% of AML patients. Rearrangements of Igλ genes have never been reported. Rearrangements of TcR-β genes, TcR-γ genes, and TcR-δ genes have been found in 7%, 5%, and 9% of AML, respectively. In this study it was not possible to demonstrate an association between the presence of a TdT^+ leukemic subpopulation and the occurrence of cross-lineage Ig or TcR gene rearrangements in AML. These rearrangements were detected in 13% (5/38) of AML with a TdT^+ leukemic subpopulation and in 19% (3/16) of TdT^- AML. Review of these data and over 400 published AML cases in which at least two different Ig and/or TcR genes had been investigated revealed that cross-lineage rearrangements of these genes concur frequently. Igκ gene rearrangements were only found in AML with rearranged IgH genes, whereas TcR-β genes and TcR-γ genes were only rearranged in combination with rearranged TcR-δ genes and/or IgH genes. Based on these data, an ordered pattern of cross-lineage Ig and TcR gene rearrangements in AML can be postulated, in which rearrangements of IgH genes or TcR-δ genes precede the other cross-

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lineage rearrangements.

INTRODUCTION

The expression of Ig and TcR molecules by lymphocytes is preceded by a series of hierarchically ordered somatic rearrangements of the variable (V), diversity (D), and joining (J) gene segments which code for these molecules (1,2). From studies on acute lymphoblastic leukemias (ALL), which can be regarded as malignant counterparts of immature lymphoid cells, it is known that during B cell development IgH gene rearrangement precedes rearrangement of Igκ and Igλ genes and that in the early stages of T cell differentiation rearrangement of TcR-δ genes is followed by rearrangement of TcR-γ genes and TcR-β genes (3-7). Although Ig and TcR molecules are only expressed by B and T lymphocytes, respectively, rearrangements of Ig and TcR genes appear not to be lineage restricted. Cross-lineage rearrangements of IgH genes have been observed in 10%-15% of T-ALL, while TcR-β, TcR-γ, and TcR-δ gene rearrangements and/or deletions have been found in about 33%, 55%, and 80% of precursor B-ALL, respectively (7-11). The occurrence of these cross-lineage rearrangements has been explained by the assumption that B and T cells use a common recombinase for gene rearrangement (12). TdT probably plays an important role during Ig and TcR gene rearrangements by adding nucleotides at the junction sites of V-(D-)J gene segments, thereby increasing the diversity of Ig and TcR molecules (2,13-15).

AML can be regarded as malignant counterparts of either cells arrested at undifferentiated stages or cells arrested at early stages of the myeloid differentiation. Rearrangement of Ig and/or TcR genes have been reported to occur in 0%-76% of AML (16-34). The precise frequency of the cross-lineage gene rearrangements in AML is difficult to calculate from data reported previously, because several studies have selected for particular subtypes of AML and in most studies only a restricted number of Ig and TcR genes have been investigated. Studies in which all Ig and TcR gene complexes (i.e. IgH, Igκ, Igλ, TcR-β, TcR-γ, and TcR-δ/α) were investigated are still lacking. Initially, an association between Ig and/or TcR gene rearrangements and the expression of the TdT in AML has been postulated (19,22,24,28), but this could not be confirmed in some recent studies (25,26,30-32). In all these studies an AML was considered to be TdT^+ if the percentage of TdT^+ cells was $\geq$10%, or even $\geq$25%. Recently, we demonstrated that TdT^+ leukemic subpopulations occur in about 75% of the AML, but that the percentage of these leukemic TdT^+ cells is $\leq$10% in 40% of AML (35).

The aim of our study was to determine the frequency of Ig and/or TcR rearrangements in AML and whether these cross-lineage gene rearrangements correlate with the expression of TdT. Fifty-four unselected AML patients were analyzed for the presence of a TdT^+ subpopulation using double marker analysis for TdT and myeloid markers (35), as well as for rearrangements and/or deletions in the IgH, Igκ, Igλ, TcR-β, TcR-γ, and TcR-δ/α genes. To determine whether Ig and TcR gene rearrangements in AML occur in an ordered fashion as found in ALL, the results of these 54 AML and over 400 cases published previously were evaluated.

MATERIALS AND METHODS

Patients and cell samples

Peripheral blood (PB) or bone marrow (BM) samples from 54 unselected AML patients were investigated. Thirteen patients were children (<18 years). Patients with a myeloid blast crisis of a chronic myelocytic leukemia were not included. In 50 patients the analyses were performed at diagnosis, in three patients at first relapse and in one patient at second relapse. The diagnosis of AML was based on cytomorphology of PB and BM smears stained for May Grünwald Giemsa and cytochemistry (Sudan black B, myeloperoxidase and α-naphtylacetate esterase). The patients were classified according to the revised French American British (FAB) criteria (36). AML which were minimally differentiated were classified as either AML-M0 or acute undifferentiated leukemia (AUL). Mononuclear cells (MNC) were isolated from BM and PB samples by Ficoll Paque (density: 1.077 g/cm^3; Pharmacia, Uppsala, Sweden) density centrifugation. Immunological marker analysis was performed on freshly isolated MNC, whereas the MNC samples for DNA analyses were frozen and stored in liquid nitrogen.

Immunological marker analysis

The results of immunological marker analysis of 38 AML samples were published previously (35). Sixteen additional AML samples were analyzed for the same markers, including the nuclear expression of TdT, the expression of the B cell markers CD19 (B4) and CD10 (VIL-A1), the T cell markers CD2 (T11), CD3 (Leu-4), and CD7 (3A1), the myeloid monocytic markers CD13 (My7), CD14 (My4), CD15 (VIM-D5), CD33 (My9), and CDw65 (VIM-2), the erythroid marker glycophorin A (GpA;VIE-G4), the platelet marker CD61 (C17), and the non-lineage specific markers CD34 (BI-3C5) and HLA-DR (L243). The rabbit anti-TdT antiserum was obtained from Supertechs (Bethesda, MD, USA); the monoclonal antibodies (McAb) B4, T11, My7, My4, and My9 were obtained from Coulter Clone (Hialeah, FL, USA); Leu-4 and L243 were obtained from Becton Dickinson (San Jose, CA, USA); VIL-A1, VIM-D5, VIM-2, and VIE-G4 were gifts from Dr. W. Knapp (Vienna, Austria); the 3A1 hybridoma was obtained from the American Type Culture Collection (Rockville, MD, USA); the McAb C17 and BI-3C5 were obtained from Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands) and Seralab (Crawley Down, UK), respectively. Immunological marker analysis was performed as described previously (37). Surface membrane markers were visualized by use of a fluorescein isothiocyanate (FITC) conjugated goat anti-mouse Ig antiserum (Central Laboratory of the Blood Transfusion Service) and evaluated with either Zeiss fluorescence microscopes (Carl Zeiss, Oberkochen, Germany) or with a FACScan flowcytometer (Becton Dickinson). A leukemia was regarded to be positive for an immunological marker if more than 20% of MNC expressed the marker. Binding of the anti-TdT antiserum was visualized by use of an FITC-conjugated goat anti-rabbit Ig antiserum (Supertechs). The surface membrane phenotype of TdT$^+$ cells was determined as described using double marker analysis for TdT and membrane markers such as CD10, CD13, CD19, CD33, and CDw65 (35,37). The TdT immunofluorescence (IF) labelings as well as the double marker analyses were analyzed with Zeiss fluorescence microscopes.

Southern blot analysis

DNA was isolated from MNC as described previously (38,39). DNA (15 μg) was digested with the appropriate restriction enzymes, obtained from Boehringer Mannheim (Mannheim, Germany) or Pharmacia. The restriction fragments were size fractionated in 0.7% agarose gels and transferred to Nytran-13N nylon membranes (Schleicher and Schuell, Dassel, Germany) (39). The probes were ^{32}P random oligonucleotide labeled.

The configuration of IgH genes was analyzed using a JH probe in *Bgl*II and in *Hin*dIII digests (39). Igκ rearrangements were detected with a Jκ probe in *Hin*dIII digests and a Cκ probe in *Bam*HI digests (39). The

TABLE 1. Summary of the results of immunological marker analysis of 54 AML patients.

		myeloid marker$^+$,TdT$^+$[a]	CD2	CD3	CD7	CD10	CD13	CD14	CD15	CD19	CD33	CD34	CDw65	HLA-DR
Total group of AML		38/54[b]	5/52	0/54	8/54	0/54	40/54	17/54	17/54	2/52	48/53	29/47	18/21	45/53
AML with Ig and/or TcR gene rearrangements														
patient	FAB diagnosis													
P.M.	AML-M0	0%	—	—	—	—	—	—	—	—	+	—	+	+
R.M.	AML-M2	0%	+	—	—	—	+	+	+	—	+	—	NT	+
M.Z.	AML-M7[c]	0%	—	—	—	—	+	—	—	—	+	—	+	—
R.S.	AML-M0	7%	—	—	+	—	—	—	—	—	+	—	NT	+
J.C.	AML-M4	8%	+	—	—	—	+	+	+	—	+	+	NT	—
A.V.	AML-M2	14%	—	—	—	—	+	—	—	—	+	+	NT	+
B.M.	AML-M0	65%	—	—	—	—	+	—	+	—	+	+	NT	+
H.B.	AUL	78%	—	—	—	—	—	—	+	—	—	+	+	+

Symbols used: +, at least 20% of the MNC expressed the marker; –, <20% of the MNC expressed the marker; NT, not tested.

a. Cells expressing both TdT and a myeloid marker (CD13, CD33 and/or CDw65).

b. At least 0.1% of the MNC was myeloid marker$^+$, TdT$^+$.

c. The majority of the blast cells were CD61$^+$.

configuration of the Igλ genes were analyzed using a Cλ probe in *Eco*RI/*Hin*dIII double digests (39).

The Cβ and Jβ2 probes in *Eco*RI digests were used to detect TcR-β rearrangements, which were confirmed using the Cβ probe in *Bam*HI digests (39). Rearrangements of TcR-γ genes were detected with a Jγ1.3 probe in *Eco*RI and *Kpn*I digests, which allows detection of all rearrangements in both Jγ1 and Jγ2 regions (39). The configuration of the TcR-δ genes was analyzed using the Jδ1 probe, Jδ2 probe, and Cδ probe. The Jδ1 probe was used in *Kpn*I, *Bgl*II, *Hin*dIII, and *Bam*HI digests, the Jδ2 probe was used in *Eco*RI and *Bam*HI digests, and the Cδ probe was used in *Bam*HI digests. These analyses allow the detection of all rearrangements and deletions in the Dδ-Jδ-Cδ gene region of the TcR-δ/α locus (39).

RESULTS

Cytomorphology

One leukemia was classified as AUL and 53 leukemias were classified as AML. According to the FAB criteria these AML were classified as M0 (n=7), M1 (n=6), M2 (n=19), M4 (n=12), M5 (n=7), M6 (n=1), and M7 (n=1).

Immunological marker analysis

The results of the immunological marker analysis of the 54 AML are summarized in Table 1. Thirty-five AML were positive for both CD13 and CD33, whereas single expression of CD13 or CD33 was found in five and 13 AML samples, respectively. The AUL (patient H.B.) was negative for these two pan-myeloid markers but positive for the myeloid marker CDw65. All AML samples were negative for CD10 and GpA. About 10% of GpA^+ cells were found in the AML classified as M6. The expression of CD61 was found on most cells of the AML-M7 (patient M.Z.). About 10% of $CD61^+$ cells were detected in two AML patients classified as M1 and M4. Thirty-eight AML samples contained myeloid $marker^+$, TdT^+, $CD10^-$ cells in percentages from 0.1% to 83% of the MNC. In the AUL sample from patient H.B. the TdT^+ cells (78% of MNC) expressed CD15, CD34, CDw65, and HLA-DR.

Southern blot analysis

The results of the Southern blot analysis of the 54 AML are summarized in Table 2. The AML are grouped based on the presence of myeloid $marker^+$, TdT^+ cells. Representative examples of IgH gene and TcR-δ gene rearrangements are given in Figure 1. In eight AML patients rearrangement of one or more Ig and/or TcR genes was seen. In seven patients the IgH genes were involved. In two of these seven cases Igκ and/or TcR gene rearrangements also occurred (Table 2). In one AML patient (R.S.) IgH genes were in germline configuration,

TABLE 2. Summary of Ig gene and TcR gene rearrangements in 54 AML patients.

Types of AML		Rearranged genes					
		IgH	Igκ	Igλ	TcR-β	TcR-γ	TcR-δ
I. no TdT$^+$ cells present (n=16)		3/16	1/16	0/16	2/16	1/16	1/16
II. 0.1%-10% of myeloid marker$^+$, TdT$^+$ cells[a] (n=24)		1/24	0/24	0/24	1/24	1/24	1/24
III. >10% of myeloid marker$^+$, TdT$^+$ cells[a] (n=14)		3/14	0/14	0/14	0/14	0/14	0/14
AML with Ig and/or TcR gene rearrangements[b]							
P.M.	TdT= 0%	R/R/R^w	G	G	R/G	R/R	D/R
R.M.	TdT= 0%	R/G	G	G	G	G	G
M.Z.	TdT= 0%	R/R/G[c]	R/R/G[c]	G	R/R/G[c]	G	G
R.S.	TdT= 7%	G	G	G	R/G	R/R	R/R
J.C.	TdT= 8%	R/G	G	G	G	G	G
A.V.	TdT=14%	R^w/R^w/R^w/R^w/R^w/G	G	G	G	G	G
B.M.	TdT=65%	R/G	G	G	G	G	G
H.B.	TdT=78%	R/R/R^w/R^w/R^w/G	G	G	G	G	G

a. Cells expressing both TdT and a myeloid marker (CD13, CD33, and/or CDw65).
b. G, germline band; R, rearranged band; R^w, weak rearranged band; D, deletion of the involved gene (segment).
c. Only 25% of AML cells were present in the MNC samples of patient M.Z., which explains the presence of the germline band in addition to the rearranged bands (Figure 1).

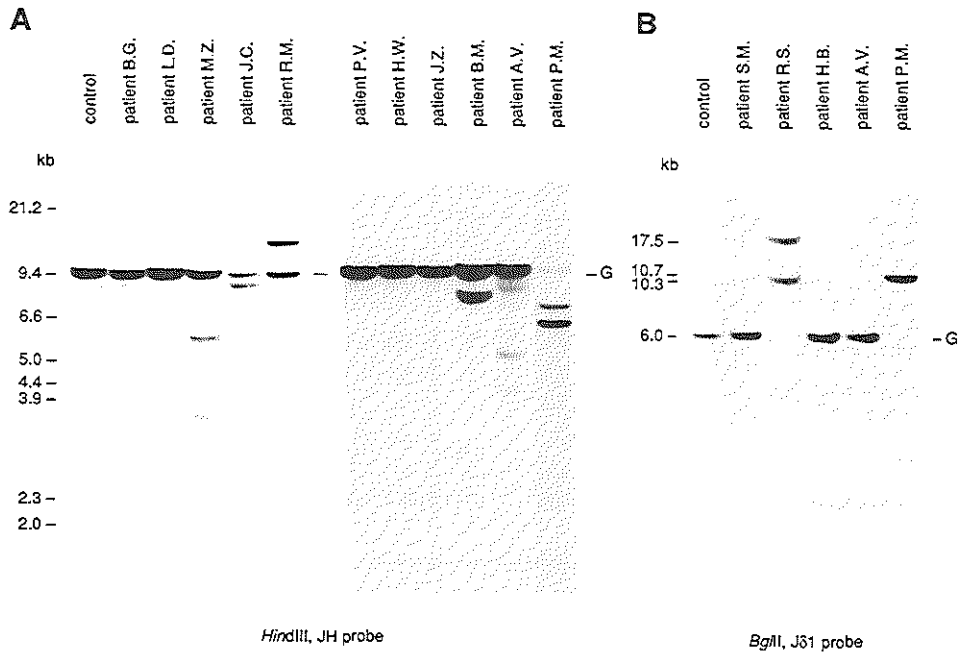

Figure 1. Southern blot analysis of the IgH and TcR-δ genes in some AML. Control DNA and DNA from AML were digested with *Hind*III (A) and *Bgl*II (B), size separated and blotted onto nylon membrane filters, which were hybridized with the ^{32}P labeled JH probe (A) and with the ^{32}P labeled Jδ1 probe (B). The sizes (in kb) of the germline bands (G), several molecular weight markers (A) and rearranged bands (B) are indicated. The patients are indicated with their initials. A: The IgH genes were rearranged in 6 of the 11 AML presented. Rearrangement of the JH gene locus on one allele was found in three patients (J.C., R.M., and B.M.) and on both alleles in one patient (M.Z.). The strong germline band in the lane of patient M.Z. is due to the presence of 75% non-leukemic cells. In patient A.V. more than two rearranged IgH gene bands of different density were visible. In patient P.M. three rearranged bands of different density were detected in *Bgl*II digests, whereas in the presented *Hind*III digest only two rearranged bands of different density were visible. This is probably due to co-migration. B: In two AML patients TcR-δ gene rearrangements were detected: patient R.S. had both alleles rearranged and patient P.M. had one allele rearranged and the other deleted (see Table 2 for details).

whereas the TcR-β, TcR-γ, and TcR-δ genes had been rearranged. Monoallelic IgH gene rearrangements were found in three AML and biallelic in one AML patient. In the other three AML samples with IgH gene rearrangements either three non-germline bands (patient P.M.) or five non-germline bands (patients A.V. and H.B.) were observed, suggesting the formation of at least two and three subclones, respectively (Figure 1).

The results of the immunological marker analysis of the eight AML patients with cross-lineage gene rearrangements are given in Table 1. Five patients were CD13$^+$ and seven CD33$^+$. Positivity for CD34 was found in four AML patients. Interestingly, the three AML patients with one or more TcR gene rearrangements were CD34$^-$.

TABLE 3. Ig and TcR gene rearrangements in AML.

Reference[a]	IgH genes	Igκ genes	Igλ genes	TcR-β genes	TcR-γ genes	TcR-δ genes
Rovigatti et al. (16)	2/14					
Ha et al. (17)	1/19	0/19	0/19			
Cheng et al. (18)	1/24	0/24		3/24		
Foa et al. (19)	10/52	3/46	0/26	1/47	4/51	
Boehm et al. (20)	3/17	0/17	0/17	3/17	(12/17)[b]	
Chen et al. (21)				0/9	0/9	
Serementis et al. (22)	9/42			10/42		
Goorha et al. (23)	2/12			0/12	0/12	
Lee et al. (24)	1/7			0/7	2/7	
Wainscoat et al. (27)	0/17			0/17	0/17	
Oster et al. (28)	10/69	1/69		3/69		
Papadopoulos et al. (29)	2/39	0/39		0/39		
Fontenay et al. (31)	10/57			4/57	3/57	8/57
This study	7/54	1/54	0/54	3/54	2/54	2/54
TOTAL (percentage)	58/423 (14)	5/268 (2)	0/116 (0)	27/394 (7)	11/207 (5) 23/224 (10)[b]	10/111 (9)

a. Only reports which describe at least seven AML cases were used for this table. AML were diagnosed according to the FAB criteria and the investigators did not mention selection criteria for inclusion in the study.

b. When the data from Boehm et al. are excluded (see text), TcR-γ gene rearrangements appear to occur in 5% (11/207) of AML cases.

DISCUSSION

This is the first AML study in which all Ig and TcR gene complexes were investigated. In 15% (8/54) of the AML patients one or more genes were rearranged. This especially concerned IgH gene rearrangements (seven cases). Rearrangement of TcR-β, TcR-δ, TcR-γ, and Igκ genes were found less frequently, whereas rearrangement of Igλ genes could not be demonstrated (Table 2). Since the first description of cross-lineage IgH gene rearrangement in a case of AML (16), the configuration of IgH genes and/or TcR-β genes of more than 400 AML patients have been reported (17-34). The other gene complexes have been investigated less frequently (17-21,23-29,31-33) and TcR-δ gene rearrangements were investigated in only one study (31). To determine the frequency of rearrangements of the various Ig and TcR genes we reviewed studies in which at least seven AML patients were investigated (16-24,27-29,31, and this study) (Table 3). Studies on a selected group of AML (e.g. TdT^+ AML, $CD7^+$ AML or AML with specific FAB morphology) were excluded (25,26,30,32-34). It was found that IgH gene rearrangements occur in 14% of AML and that Igκ gene rearrangements can be found in 2% of AML cases (Table 3). As in our study, the rearrangement of Igλ genes have never been reported (17,19,20). In 7% of AML patients rearrangement of TcR-β genes was found (Table 3). The frequency of TcR-γ gene rearrangements was 10% (19-21,23,24,27,31). However, most of the rearranged samples were published by Boehm et al., who found rearrangement of TcR-γ genes in 70% (12/17) of their AML patients (20). In all other studies

together the frequency of TcR-γ gene rearrangement was only 5% (11/207) (Table 3). This discrepancy may be explained by the limited combinatorial repertoire of the TcR-γ genes, which results in a restricted number of rearranged bands when polyclonal T cells are present (39-41). Although Boehm et al. (20) remark that contaminating T cells were probably not responsible for the detected rearranged bands, they could not exclude this possibility. Rearrangement and/or deletion for TcR-δ genes, which are located within the TcR-α gene complex (42,43), were demonstrated in eight patients by Fontenay et al. (31) and in two patients in this study, which together form 9% of the AML patients tested. Analysis of a larger group of AML patients is needed to determine the precise frequency of TcR-δ gene rearrangement in AML.

We could not find a correlation between a specific immunophenotype of AML and the occurrence of cross-lineage gene rearrangements. The only remarkable finding was the lack of CD34 expression in AML with one or more rearranged TcR genes. According to Fontenay et al. (31) TcR-δ gene rearrangements in AML occur predominantly in immature myeloid leukemias exhibiting immunophenotypic "lineage promiscuity", of which the most striking feature was the frequent expression of CD10. Our two AML patients with TcR-δ gene rearrangements were both classified as AML-M0, but CD10 expression was not found.

Controversy exists about the relationship between the expression of TdT in AML and the occurrence of cross-lineage Ig and TcR gene rearrangements. Four initial reports claimed such an association, as 82% (33/40) of the TdT^+ AML had rearranged Ig and/or TcR genes, whereas such rearrangements were found in only 3% (4/130) of the TdT^- AML patients (19,22,24,28). When the results from seven additional studies are combined this association cannot be confirmed (16,25,26,29,30,32,33). In these seven studies Ig and/or TcR gene rearrangements, were found in 18% (11/61) of the TdT^+ cases and in 13% (9/69) of the TdT^- cases. The number of genes studied and the criteria to consider an AML to be TdT^+ were roughly identical. In a study on AML patients, using double marker analysis for both a myeloid marker and TdT, we could identify a TdT^+ leukemic subpopulation in about 75% of the AML, but we found that the percentage of TdT^+ AML cells was low (≤10%) in 40% of patients (35). In this study we investigated the occurrence of cross-lineage gene rearrangements in 16 TdT^- AML patients and in 38 AML patients with a TdT^+ leukemic subpopulation (ranging from 0.1% to 83% of MNC). The incidence of cross-lineage gene rearrangements were comparable in both groups: 19% (3/16) in TdT^- AML and 13% (5/38) in AML with a TdT^+ subpopulation. If a 10% cut-off percentage for TdT positivity was used, 21% (3/14) of the TdT^+ AML were found to contain an IgH gene rearrangement (Table 2). This percentage is in line with the results of the seven studies mentioned earlier (16,25,26,29,30,32,33).

TdT plays an important role during lymphoid differentiation by inserting nucleotides at the junction sites of rearranging Ig and TcR genes, but this enzyme is not essential for gene rearrangement (12-15). This may explain why not all AML with rearranged Ig and/or TcR genes express TdT. Sequence analysis of the junctional regions in rearranged genes of TdT^- AML may demonstrate whether these cells indeed lacked any TdT activity during gene rearrangement. On the other hand, the finding of germline genes in most TdT^+ AML patients raises the question of whether extra nucleotide insertion may occur outside Ig and TcR genes. An intriguing speculation is that TdT may add extra nucleotides at DNA breakpoints such as

TABLE 4. Coincidence of Ig and/or TcR gene rearrangements in AML.

Groups of AML	The indicated groups of AML also have rearranged and/or deleted Ig and TcR genes in the following frequencies[a]:					
	IgH genes	Igκ genes	Igλ genes	TcR-β genes	TcR-γ genes	TcR-δ genes
AML with IgH rearrangement		18 (6/33)	0 (0/10)	29 (20/68)	25 (9/36)	41 (7/17)
AML with germline IgH genes		0 (0/225)	0 (0/92)	3 (13/395)	2 (4/206)	3 (3/94)
AML with Igκ rearrangement	100 (6/6)		0 (0/3)	50 (2/4)	0 (0/4)	0 (0/1)
AML with germline Igκ genes	11 (27/252)		0 (0/97)	3 (8/229)	5 (5/96)	4 (2/53)
AML with germline Igλ genes	10 (10/102)	3 (3/100)		4 (3/81)	2 (2/80)	4 (2/54)
AML with TcR-β rearrangement	61 (20/33)	20 (2/10)	0 (0/3)		54 (6/11)	86 (6/7)
AML with germline TcR-β genes	11 (48/430)	1 (2/223)	0 (0/78)		2 (6/236)	4 (4/104)
AML with TcR-γ rearrangement	69 (9/13)	0 (0/5)	0 (0/2)	50 (6/12)		100 (5/5)
AML with germline TcR-γ genes	12 (27/229)	4 (4/95)	0 (0/78)	2 (5/235)		5 (5/106)
AML with TcR-δ rearrangement	70 (7/10)	0 (0/2)	0 (0/2)	60 (6/10)	50 (5/10)	
AML with germline TcR-δ genes	10 (10/101)	2 (1/52)	0 (0/52)	1 (1/101)	0 (0/101)	

The data are derived from references 17-19, 21-34, and from this study (Table 2). Data from the study of Boehm et al. (20) were not included (see text).
a. Values given as percentage (number/total).

chromosome translocations, inversions and deletions. Such a tumor associated expression of TdT would explain the rare occurrence of myeloid$^+$, TdT$^+$ cells in normal BM samples (44,45).

In three of the seven IgH gene rearrangements in our AML group this rearrangement involved only one allele. Combined data reported previously are comparable with our results and revealed a frequency of 44% (19/43) of monoallelic IgH gene rearrangements in AML (16-20,25,26,29-32,34). Interestingly, in T-ALL with IgH gene rearrangements this frequency is much higher (~85%) (7,9), whereas monoallelic IgH rearrangements occur in only 15%-20% of the precursor B-ALL (4,7). In three other AML patients we detected more than two rearranged bands, which suggests the formation of subclones (46).

A remarkable finding in our group of patients was the concurrence of Ig gene and/or TcR gene rearrangements in three AML. IgH gene rearrangement was the only rearrangement which occurred in an isolated manner. To investigate whether concurrence of cross-lineage gene rearrangements occurs frequently in AML and whether these rearrangements occur in an ordered fashion, we reviewed AML patients in which the configuration of at least two rearranging genes had been investigated (17-19,21-34, and this study). The results of this comparison are summarized in Table 4. Rearrangements of TcR genes occurred more often in AML with rearranged IgH genes than in AML with germline IgH genes. Furthermore, rearrangements of the different TcR genes concurred frequently. These data support the hypothesis for the existence of a common recombinase for Ig and TcR genes (12). However, as in ALL, the rearrangements seem to occur in a non-random ordered fashion (3-7). Igκ gene rearrangements were only demonstrated in AML with rearranged IgH genes. It was remarkable to find that in two out of four AML with a rearranged Igκ gene the TcR-β genes were also rearranged (28 and this study). Within the group of AML which contained germline TcR-δ, genes only one leukemia had a rearrangement for another TcR gene, i.e. TcR-β gene. This AML patient (M.Z. of this study) had rearranged IgH genes and Igκ genes. Based on these results, an ordered pattern of cross-lineage Ig and TcR gene rearrangements in AML can be postulated, in which rearrangements of IgH or TcR-δ genes precede the other cross-lineage rearrangements.

Finally, it is intriguing to consider the possibility of using the polymerase chain reaction mediated amplification of the junctional regions of rearranged Ig and TcR genes for the detection of minimal residual disease in AML patients with cross-lineage gene rearrangements (47-49). However, it should be emphasized that oligoclonality at diagnosis, which we could demonstrate in three out of the seven patients with IgH gene rearrangements, will hamper the application of this technique and may lead to false negative results (46,50).

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CHAPTER 5

GENERAL DISCUSSION

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Acute myeloid leukemia (AML) is a heterogeneous disorder with a marked patient to patient variation in clinical presentation, cytomorphology, cytogenetics, response to therapy, and prognosis. In contrast to most acute lymphoblastic leukemias (ALL), also a marked immunophenotypic heterogeneity is found in the majority of AML patients. We performed extensive immunological marker studies to determine the extent of the immunophenotypic heterogeneity in AML. The main objective of this study was to recognize specific subgroups of AML patients and to characterize immature subpopulations of AML cells which might be useful for monitoring the disease during follow-up.

5.1 ASSOCIATIONS BETWEEN IMMUNOPHENOTYPE AND KARYOTYPE

As has been described in Chapter 1, alterations in critical genes play a central role in the multistep process of leukemogenesis. The observation that most of the non-random chromosome abnormalities are associated with specific clinical and phenotypical features has given additional evidence for the biological importance of cytogenetic abnormalities in AML. So far, most data on associations between genotype and phenotype of AML concerned combinations of specific karyotypic aberrations and morphological subtypes of AML according to the French American British (FAB) classification (see also Chapter 3.1). However, it is conceivable that, like in ALL, cytogenetic aberrations in AML are associated with specific immunophenotypes as well. Literature data on such associations are scarce and most cases reported concern associations between absence or presence of a specific marker and a karyotypic aberration, such as CD19 expression in AML with t(8;21)(q22;q22) (1-3) (see also Chapter 3.1). Another well-defined association between a karyotypic aberration and immunophenotype concerns the expression of the $CD9^+$, $CD13^+$, $CD15^-$, $CD33^+$, $HLA\text{-}DR^-$ phenotype in AML-M3 or M3 variants with t(15;17)(q22;q21) (4,5). In contrast to most other types of AML, the majority of AML cases with t(15;17) consist of homogeneous leukemic cell populations. This homogeneity is in line with the assumption that these leukemias are probably derived from a more mature myeloid progenitor cell than other types of AML and that the maturation arrest seems to be complete (6). However, addition of retinoic acid (RA) results in further maturation, which indicates that the arrest in AML with t(15;17) can be overruled (7-9). This is illustrated by the observation that RA induces maturation of the $CD15^-$ AML cells into $CD15^+$ cells (9). It has been proposed that AML with t(15;17) should be considered as a leukemia with unique clinical and

biological characteristics, which differ from all other AML types (10,11).

The lack of associations between genotype and immunophenotype in other types of AML is probably related to the intraclonal heterogeneity. Most laboratories do not use double immunofluorescence (IF) staining but perform single color immunological marker analysis for immunophenotyping of AML. Single color stainings are sufficient for immunophenotyping of homogeneous cell populations, but have limited value in case of heterogeneous cell populations, especially if rigid cut off values of 15% to 25% positivity are used. Therefore detailed immunophenotyping of the various subpopulations within heterogeneous cell populations needs multiparameter analysis with double IF stainings. Our results presented in Chapter 3.3 clearly demonstrate that double IF staining allows the identification of various subpopulations in AML-M4Eo with inv(16)(p13q23). Although the proportion of these subpopulations differed, each of the eight AML-M4Eo tested contained identical immature and mature subpopulations. Interestingly, not only the presence or absence of certain markers but also the fluorescence intensity of most markers was comparable, indicating that antigen density might be used as an additional parameter. In addition, heterogeneity in "morphological" parameters, such as phase contrast microscopy or light scatter profiles, can be included in multiparameter analysis of AML (12,13).

As indicated above, multiparameter analysis of AML with t(8;21) revealed a unique $CD19^+$ immunophenotype (3). In addition to the weak CD19 expression, we have also found weak expression of the pan-myeloid markers CD13 and CD33 but strong expression of CDw65. Furthermore AML with t(8;21) contain immature $CD34^+$, $HLA\text{-}DR^+$, partly terminal deoxynucleotidyl transferase $(TdT)^+$ cells and strong positivity for the CD15 antigen (ref.13; Adriaansen et al., unpublished results). It has been suggested that expression of the CD19 antigen in AML with t(8;21) reflects transformation of a multipotent stem cell and that such leukemias should be regarded as hybrid leukemias (2). However, no evidence of B cell commitment was found in these types of AML (1-3).

Future studies should clarify whether AML with other karyotypic abnormalities also have a consistent immunophenotype. As indicated in Chapter 3.2, AML with t(6;9) may be associated with a $CD13^+$, $HLA\text{-}DR^+$, TdT^+ phenotype. However, one of our two cases tested expressed CD33 while the other AML case was $CD33^-$. Based on the finding that t(6;9) can occur in different FAB types of AML and even in some myelodysplastic syndromes, it has been suggested that the translocation event occurs in an uncommitted stem cell (14,15). It is conceivable that maturation is more variable in AML with t(6;9) as well as in other types of AML, in which the oncogenic event is assumed to occur in an uncommitted multipotential myeloid stem cell, such as in AML with chromosome 5 or 7 abnormalities, or AML with 11q23 aberrations (16). Finally, it should be noticed that heterogeneity between AML with (apparently identical) chromosome aberrations may be determined by differences in the precise location of the breakpoints, such as recently described for $5q^-$ aberrations (17).

5.2 DETECTION OF IMMATURE SUBPOPULATIONS IN AML

By use of multiparameter analysis, in particular double IF stainings, we could recognize several immature and mature subpopulations in most AML (see Chapter 4.2). Markers which were expressed by immature cells include CD7, CD34, and TdT as well as pan-myeloid markers CD13, CD33, and often CDw65. Our results are in line with other studies, in which CD7, CD34, and/or TdT have been associated with either immature types of AML or immature subpopulations within AML (see Chapter 2.2). Additional markers for immature leukemic cells might be useful to further differentiate within the immature AML cell populations.

Recently, the monoclonal antibody (McAb) YB5.B8 was found to recognize the human c-kit protein (18-20). In the last year two other groups reported a McAb (17F11 and SR-1) against this antigen (21-23). The *c-kit* proto-oncogene, which is localized on chromosome 4q11-q12, encodes a 145-160 kDa transmembrane tyrosine kinase (24-26). The protein was found to function as receptor for the stem cell factor (SCF) (see also Chapter 1). Both YB5.B8 and SR-1 McAb can inhibit binding of SCF to blast cells, although the two McAb were found to identify distinct epitopes of the c-kit protein (20,22). Expression of the c-kit antigen was found on 1%-5% of the mononuclear cells in normal bone marrow (BM) samples (20-22). These c-kit positive cells have a blastic morphology. Double IF staining revealed that in normal BM 40%-60% of the c-kit$^+$ cells expressed CD34, whereas 50%-75% of the CD34$^+$ cells were c-kit$^+$ (21,23). Colony assays demonstrated that CD34$^+$, c-kit$^+$, HLA-DR$^-$ cell populations contain more primitive progenitor cells than CD34$^+$, c-kit$^+$, HLA-DR$^+$ cell populations (23).

Initially, about 30% of the AML cases were reported to be positive for YB5.B8 (18,19). Especially those AML patients with poor prognosis were found to be positive for this McAb (18,19). However, an increased level of c-kit mRNA could be identified in the majority of AML cases (20). Furthermore, 87% of the AML tested were positive for the McAb 17F11, but more than half of the positive samples contained only a minor c-kit$^+$ subpopulation (< 10%) (21). The results of the mRNA study suggested that AML-M3 and AML-M5b are c-kit negative (20). However, it should be tested at the single cell level whether low levels of mRNA are caused by a low c-kit expression in all AML cells or a low number of c-kit$^+$ cells. The ALL cases tested so far were negative for the c-kit antibodies (21).

By use of double IF staining Bühring et al. showed that the majority of c-kit$^+$ AML blasts coexpressed CD34 (21). In addition, CD34$^+$, c-kit$^-$ cell populations were demonstrated in all AML, whereas CD34$^-$, c-kit$^+$ cells were only found in 3 out of 26 samples tested. At present it is not known whether CD34$^+$, c-kit$^-$ cells or CD34$^-$, c-kit$^+$ cells represent the most immature AML subpopulation. We recently obtained the 17F11 McAb and performed double IF stainings for c-kit and several other antigens in six AML. The results are summarized in Table 1 and Figures 1 and 2. In five out of the six AML, we could identify a c-kit$^+$ cell population. The only c-kit negative AML concerned an AML-M0 (patient R.S.). By use of double IF stainings we could confirm the observations that the far majority of c-kit$^+$ AML

TABLE 1. Analysis of c-kit expression in PB of six AML patients at diagnosis[a].

Patients	M.B.	A.K.	T.B.	N.A.	B.M.	R.S.
Age;sex	(10;F)	(26;M)	(59;M)	(46;M)	(76;F)	(18;M)
FAB type	M4Eo	M4Eo	M2	M2	M0	M0
Karyotype	inv(16)	inv(16)	t(8;21)	t(8;21)	$5q^-$,-7[b]	t(9;9)
TdT	0.1(88)[c]	0.6(79)	10(15)	11(66)	37(35)	7(0)
c-kit(17F11)	37	42	9	42	13	<1[d]
Immunophenotype of c-kit$^+$ cells						
CD2 (T11)	61	30	<1	<1	<1	
CD3 (Leu-4)	<1	<1	<1	<1	<1	
CD7 (CLB-CD7)	<1		<1	<1	<1	
CD13 (My7)	98	99	96	68	80	
CD14 (My4)	<1	<1	<1	<1	<1	
CD15 (Leu-M1)	36		<1	<1	40	
CD19 (B4)	<1		69	66	<1	
CD33 (My9)	42	74	44	53	81	
CD34 (HCPA-2)	89	98	87	96	87	
HLA-DR (L243)	71	82	84	99.5	98	

a. Figures represent percentages positivity per MNC (in case of TdT and c-kit) or percentages positivity per c-kit$^+$ cells.
b. Complex tetraploid karyotype with among others $5q^-$ and -7.
c. Between brackets are indicated percentages of c-kit$^+$ per TdT$^+$ cells as determined by double IF staining.
d. Due to the virtual absence of c-kit expression in patient R.S., no reliable analysis of the c-kit$^+$ cells could be performed.

cells expressed CD34. These cells were also CD13$^+$, CD33$^+$, and HLA-DR$^+$. Interestingly, in two CD2$^+$ AML-M4Eo with inv(16) and in two CD19$^+$ AML-M2 with t(8;21), the c-kit$^+$ cells were positive for CD2 and CD19, respectively. This implies that the characteristic CD2 and CD19 expression in these two AML types are also present in the immature AML cell fractions. Furthermore, coexpression of c-kit and TdT could be determined in all five c-kit$^+$ AML, which further supports our hypothesis, that TdT$^+$ subpopulations represent immature AML cells (Chapter 4.2). These data illustrate that c-kit antibodies are valuable for phenotyping of immature subpopulations within AML.

5.3 WHY IS TdT EXPRESSED IN AML?

In our studies we could demonstrate myeloid marker$^+$, TdT$^+$ subpopulations in ~75% of AML cases (Chapter 4.2). In contrast to the homogeneous expression of TdT in most ALL, TdT expression in AML is often restricted to immature subpopulations of the leukemia. Myeloid marker$^+$, TdT$^+$ cells are rare in normal BM and not detectable in normal peripheral blood (PB) (Chapter 4.1). It might be hypothesized that TdT$^+$ AML cells represent a malignant expansion of the rare myeloid marker$^+$, TdT$^+$ cell population in BM. On the other hand, it is possible that expression of the TdT gene is dysregulated in these AML.

The TdT gene is located on chromosome 10q23-q24 (27,28), a locus which is probably not involved in acute leukemia. High levels of TdT expression are found in normal precursor

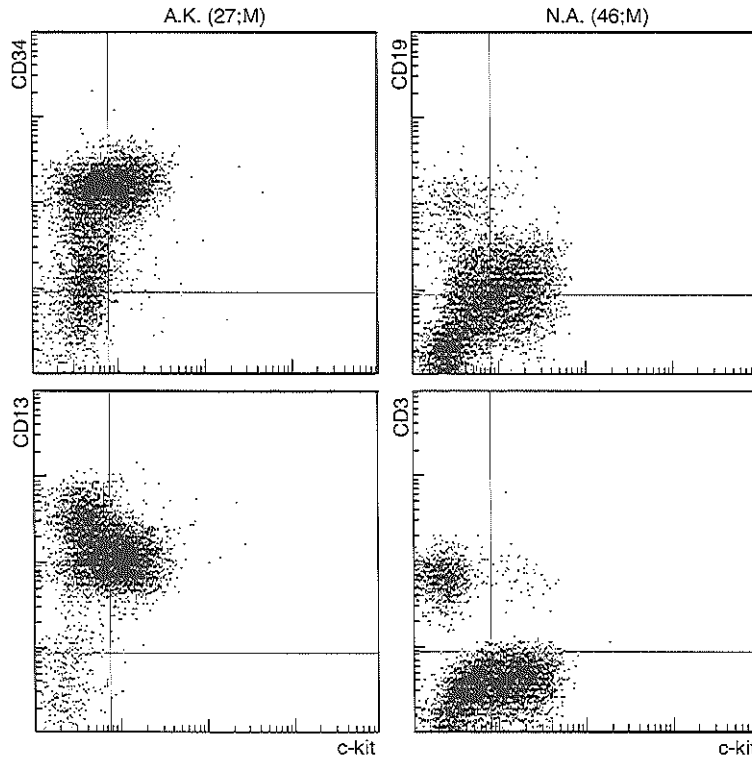

Figure 1. Dot plot analysis of four double IF staining experiments on PB cells from two different AML patients at diagnosis to determine the phenotype of c-kit$^+$ AML cells. **Left:** patient A.K. having an AML-M4Eo with inv16(p13q23), c-kit (17F11 + goat anti-mouse immunoglobulin (GαMIg) FITC), CD34 (HPCA-2 PE) (above) and c-kit (17F11 + GαMIg FITC), CD13 (My7 PE) (below). **Right:** patient N.A. having an AML-M2 with t(8;21)(q22;q22), c-kit (17F11 + GαMIg FITC), CD19 (Leu-12 PE) (above) and c-kit (17F11 + GαMIg FITC), CD3 (Leu-4 PE) (below). See also Table 1.

lymphoid cells and their malignant counterparts. TdT is a template independent DNA polymerase (29), which adds extra nucleotides at the junctional regions of Ig and TcR genes during the rearrangement processes (30-32).

It is not precisely known, how transcription of the TdT gene is regulated. The TdT gene lacks TATA boxes, but an initiator (Inr) motif has been identified which probably functions as a transcription control element (33). It has been postulated that the Inr motif is unique for the transcription of genes which require strict activation and inactivation during cellular differentiation (33). The expression of the TdT gene in precursor lymphoid cell lines can be influenced by use of phorbol esters, such as phorbol 12-myristate 13-acetate (PMA) (34-36). Addition of PMA results in a rapid but reversible decline in TdT transcription and TdT enzyme activity (34-36). This PMA-mediated reduction can be blocked by pretreatment of the cells with protein kinase C (PKC) inhibitors, such as H7 and H1004, implying that PKC is

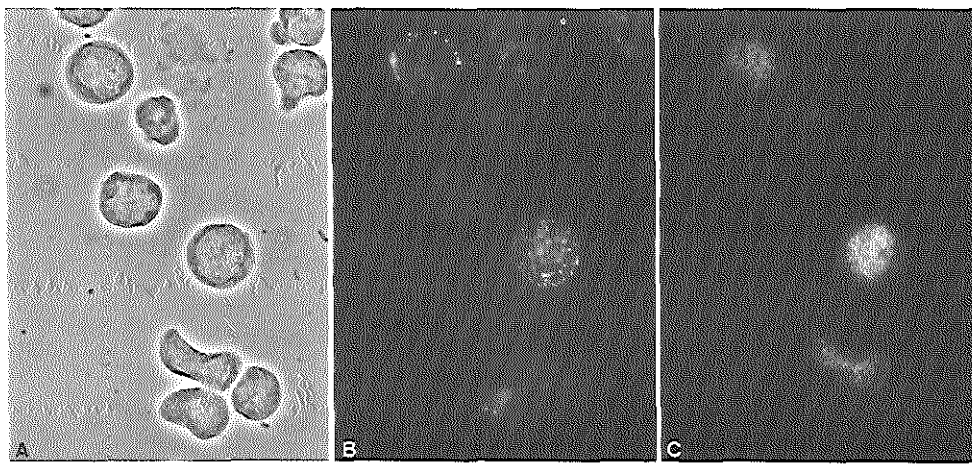

Figure 2. Double IF staining fot c-kit and TdT on PB cells from patient N.A. at diagnosis. **A**: phase contrast morphology; **B**: c-kit (17F11) positive cells (TRITC labeled); **C**: TdT positive cells (FITC labeled). Two of the three TdT^+ cells are c-kit^+.

probably involved in down regulation of TdT expression in lymphoid cells (34). We added the PKC inhibitors H7 and H1004 to the culture medium of the HL-T cell line, which is derived from the promyelocytic cell line HL-60 and contains a myeloid $marker^+$, TdT^+ subpopulation (37). Subsequently we observed both a higher expression of TdT per cell and a slight increase of the proportion of myeloid $marker^+$, TdT^+ cells (unpublished observations). These data suggest that also in myeloid cells PKC is involved in the regulation of TdT expression.

We investigated whether the expression of TdT in AML was related to the incidence of so-called cross-lineage Ig or TcR rearrangements. In our AML group as well as in a review of literature data we could not demonstrate such an association (Chapter 4.4). TdT activity in ALL not only mediates random nucleotide insertion in the junctional region of Ig and TcR genes, but also in fusion regions of chromosome breakpoints, especially in case of chromosome aberrations involving Ig and TcR genes (38-40). This implies that TdT is active during the development of the chromosome aberration and that nucleotide insertion is not restricted to junctional regions of Ig or TcR genes. Although it may be hypothesized that TdT activity during the translocation event in TdT^+ AML could result in addition of extra nucleotides at fusion regions of the chromosome breakpoints, such an effect has not been demonstrated so far. In close collaboration with Von Lindern and collagues, it was investigated whether nucleotide insertion could be found at the *dek-can* fusion regions in two AML with t(6;9) and at the *set-can* fusion region in an AML-M0 case with t(9;9) (41,42). One AML with t(6;9) and the AML-M0 case were investigated for the presence of myeloid $marker^+$, TdT^+ subpopulations and were both found to be positive (patient 2 Chapter 3.2 and patient R.S. Chapter 4.2). In the translocation breakpoints investigated we could not

demonstrate any random nucleotide insertions (42). These results suggest that TdT was not active during the translocation event in these patients or that TdT was not capable of adding nucleotides in this type of breakpoint fusion region.

5.4 DETECTION OF MINIMAL RESIDUAL DISEASE

The results of our study demonstrate that immunological marker analysis is not only useful to obtain insight in the immunophenotypic heterogeneity of AML at diagnosis, but that it is also a powerful tool to detect minimal (residual) disease during follow-up (see Chapter 4.3). Although 60% to 80% of AML patients achieve complete remission (CR), the majority of patients develop relapse and die of their disease (Chapter 1). Apparently, low numbers of leukemic cells persist, although they are undetectable by conventional cytomorphological techniques, due to their detection limit of 10^{-1} to 10^{-2} (10 to 1 cells between 100 normal cells).

Development of methods to detect residual disease has become an important goal in leukemia research. Such methods may not only be used to predict a relapse but also to monitor the effectiveness of treatment. The latter is important because new treatment strategies are currently developed to improve survival rates of AML patients. Several approaches to detect minimal residual disease (MRD) in AML have been proposed (see also Chapter 4.3). Table 2 summarizes the methods which seem to be most promising.

Immunological marker analysis

The double IF staining technique used in Chapter 4.3 allows detection of one myeloid marker$^+$, TdT$^+$ cell among 10,000 or even 100,000 normal cells (detection limit: 10^{-4} to 10^{-5}) (Table 2). This technique is applicable for the detection of residual disease in the majority of AML cases, because myeloid marker$^+$, TdT$^+$ subpopulations occur in ~75% of cases (Table 2). Our results show that a relapse of a TdT$^+$ AML is preceded by a gradual increase of myeloid marker$^+$, TdT$^+$ cells during a period of 14-38 weeks. Based on the data about c-kit expression on normal BM cells and AML cells, it might be interesting to use the c-kit antigen in MRD detection. However, normal values for coexpression of c-kit and other antigens (such as TdT) should be obtained first.

In addition to our double IF staining method, multiparameter flow cytometric analysis has been suggested for MRD detection (12,13,43). It was found that in the majority of AML patients the leukemic cells exhibit a unique phenotypic profile based on "aberrant" antigen expression and typical light scatter profiles (Table 2) (12,13,43). This might enable discrimination of leukemic cells from their normal counterparts. Despite promising results in mixing experiments (43), the application of multiparameter flow cytometry for the detection of MRD in clinical practice is limited. First of all, for each "aberrant" phenotype one should determine

TABLE 2. Techniques which enable detection of minimal residual disease in PB and BM of AML patients.

Techniques	Detection limits	Applicability
Immunological marker analysis		
- myeloid marker, TdT double IF staining	10^{-3} (BM) to 10^{-4} (PB)	~75% of AML
- multiparameter flow cytometry (scatter pattern and double or triple labeling of membrane markers)	10^{-2} to 10^{-3}	30%-70% of AML
- immunological marker analysis of *in vitro* cultured cells	unknown	some AML
Cytogenetics	10^{-1} to 10^{-2} (dependent on *in vitro* growth of AML cells)	all AML with a microscopically detectable numerical or structural chromosome aberration
Fluorescent in situ hybridization (FISH)	10^{-1} to 10^{-2} (dependent on type of chromosome aberration)	AML with well-known numerical or structural chromosome aberration, for which DNA probes are available (e.g. -5, -7, +8)
PCR techniques		
- junctional regions of rearranged Ig and TcR genes (DNA level)	10^{-3} to 10^{-6}	10%-15% of AML
- chromosome aberrations at mRNA level [e.g. t(9;22), t(15;17), and t(6;9)]	10^{-4} to 10^{-5}	5%-10% of AML

the occurrence of such cells in normal BM and PB. Comparable to our study on the occurrence of myeloid marker$^+$, TdT$^+$ cells in normal samples (Chapter 4.1), these should include cell samples from healthy volunteers as well as non-AML patients under treatment. Secondly, flow cytometry excellently enables the analysis of phenotypic markers of cell populations but this technique is not able to perform analysis at the single cell level. These combined limitations will result in detection limits (10^{-2} to 10^{-3}), which are not as low as the detection limit of the myeloid marker, TdT double IF staining technique with microscopic analysis.

AML cells can be cultured *in vitro* and clonogenic cells can subsequently be analyzed. It is obvious that for the detection of MRD this method is highly dependent on the *in vitro* growth properties of the residual AML cells and the growth of normal BM cells. It has been found that cultured AML cells can be distinguished from normal cultured cells by use of immunological marker analysis (Table 2) (44). A major problem for the application of this method is that an extensive study on the immunophenotype of non-leukemic cultured BM cells as well as the clonogenic AML cells is required in each individual patient.

Detection of clonal chromosome aberrations or rearrangements

Several other techniques for MRD detection use clonal cytogenetic aberrations or chromosome rearrangements to distinguish AML cells from normal cells. These tumor specific markers can be studied at karyotypic, DNA, mRNA, or protein level (Table 2).

According to a recent study karyotypic analysis of BM cells during CR can be used to monitor some AML patients (45). Despite the occurrence of a high false negative rate, i.e. the detection of only normal diploid metaphases in patients who subsequently relapsed, it was found that detection of the leukemic karyotype during CR was highly predictive for an imminent relapse. Especially in patients with a "favourable" cytogenetic aberration, such as inv(16) and t(8;21), karyotypic analysis proved to be useful for the detection of MRD (45). Depending on the karyotypic aberration, residual AML cells were demonstrated 10-50 weeks before cytomorphological relapse (45).

In AML numerical chromosome aberrations occur relatively frequently (see Chapter 3.1). The technique of fluorescent *in situ* hybridization (FISH) permits enumeration of specific chromosomes per cell and it can be used to detect some specific structural aberrations (46-49). The technique can be applied on both interphase and metaphase cells. For the detection of MRD in AML with trisomies or monosomies the FISH technique could be useful, although data about the occurrence of "aberrant" cells in normal BM are lacking. At present, the precise detection limit of the FISH method is not known, but has estimated to be 10^{-1} to 10^{-2} (Table 2). Most probably the detection limit of the FISH technique depends on the type of aberration, e.g. in case of trisomy the FISH technique is more sensitive than in case of monosomy.

Molecular biological techniques have enabled the study of gene rearrangements at DNA and mRNA level. The detection limit by Southern and Nothern blotting techniques is usually not much lower than 5% (50). However the polymerase chain reaction (PCR) technique has proven to be a highly sensitive method to detect low frequencies of specific DNA or mRNA sequences, with detection limits of 10^{-4} to 10^{-5} (50,51). At present in AML two types of clonal specific DNA or mRNA sequences may be used as target for PCR-mediated MRD detection, i.e. cross-lineage rearrangements of Ig or TcR genes and genes involved in specific chromosome translocations (Table 2) (51).

As has been discussed in Chapter 4.4 cross-lineage rearrangements of Ig or TcR genes occur in about 15% of AML. This especially concerns IgH gene rearrangements. Therefore, in principle the junctional regions of rearranged IgH genes can be used as PCR targets for MRD detection. However, as has been emphasized in Chapter 4.4, the application of the PCR technique may be hampered by the relatively high frequency of oligoclonal IgH gene rearrangements in AML patients.

So far, three translocations in AML have been cloned and the breakpoints have been identified, i.e. t(9;22)(q34;q11), t(15;17)(q22;q21), and t(6;9)(p23;q34) (see also Chapter 3.1) (41,52-58). In all three translocations the breakpoints are spread over relatively large areas, which hampers PCR analysis at the DNA level (41,54,59,60). However, tumor-specific hybrid

transcripts are produced by the fusion genes in these AML, which can be used as target for the PCR analysis after reverse transcriptase into cDNA (Table 2) (41,54,56,57,59,61-64). In t(15;17) two chimeric mRNAs are generated as consequence of the reciprocal translocation, i.e. *PML/RAR-α* and *RAR-α/PML* (see also Chapter 3.1) (56,57,64,65). The former transcript is expressed in all AML-M3 cases, whereas the other transcript is found in ~75% of the cases (64,65). Most data on follow-up of leukemia patients by use of PCR analysis have been obtained from studies in patients with chronic myeloid leukemia and t(9;22) (reviewed in 66,67). Much have been learned from these studies about technical aspects of the PCR technique, especially about preventing false positive results due to contamination with minute amounts of tumor specific mRNA and/or PCR products. The value of the PCR technique for the detection of residual disease in AML is under study. The first reports on a small number of patients indicate that it is possible to detect low numbers of AML cells (62,64,68). In one AML-M3 patient it was possible to detect a relapse 3 months before clinical appearance (64).

Hybrid-genes might encode for a tumor specific fusion protein. In principle, antibodies can be produced against the tumor specific epitopes, e.g. such as in case of the *bcr-abl* protein in AML with t(9;22) and the *dek-can* protein in AML with t(6;9) (41,69-71). Such antibodies might be useful for the detection of low numbers of AML cells, but reports on this type of application are still lacking.

All together these data illustrate the promising results of using tumor specific chromosome breakpoints or their products as targets for the detection of MRD. However, the three well-defined translocations mentioned above occur in less than 10% of AML. It might be expected that the precise breakpoints of other chromosome aberrations will be identified in the near future. Nevertheless, it can be anticipated that the PCR technique can be applied in only a part of AML cases (Table 2), since reciprocal translocations, inversions and deletions resulting in tumor specific fusion genes occur in only ~25% of AML (Chapter 3.1).

Comparison of methods for detection of MRD

Several methods to detect MRD in AML have been proposed (Table 2). However, it should be remarked that at present for most methods large prospective follow-up studies to evaluate the value of the approaches have not yet been performed. Data published so far as well as the results of our study indicate that MRD detection in AML is possible and that a relapse can be predicted several months before cytomorphological/clinical appearance. At the moment, there is not a single method which can be applied in all AML patients. Therefore, it is likely that a combination of techniques will be needed to monitor AML patients (Table 2).

In general, immunophenotypic methods for the detection of MRD in AML can be applied in the majority of cases and allow quantification of the tumor burden. However, in AML these methods are not completely tumor specific. PCR methods which use specific

chromosome rearrangements or their products as target for MRD detection can only be applied in a small subgroup of AML. Quantification of PCR-mediated MRD detection at the mRNA/cDNA level might be possible by use of recently published methods with internal standard mRNA (72,73). However, it should be remarked that the amount of PCR product is also dependent on the extent of transcription per cell, the quality of the isolated mRNA and the efficiency of the reverse transcriptase step.

In our study we consistently detected a relatively high number of myeloid marker$^+$, TdT$^+$ cells during CR in those AML patients who are in first continuing CR according to cytomorphological criteria. We speculated that these cells represent residual dysplastic AML cells. The leukemic origin of these cells might be proven in a part of the cases by the combined use of the PCR technique and the double IF staining technique. Alternatively, techniques which enable *in situ* detection of a leukemia-specific clonal marker, such as chromosome aberrations or Ig and/or TcR gene rearrangements might be useful (74,75). However, it is not clear whether these methods as well as some recently developed protocols for *in situ* detection of PCR products can be combined with detection of TdT (76).

5.5 CONCLUSION

It is concluded that immunological marker analysis, especially multiparameter analysis, is a powerful tool to phenotype the various subpopulations in heterogeneous disorders like AML. Such studies give insight in the differential maturation arrest of the various AML types. The recognition and characterization of immature subpopulations within each AML clone is important for the development of immunophenotypic methods for MRD detection. Application of methods for MRD detection in AML patients during follow-up might be used to adjust remission and relapse criteria, to monitor the effectiveness of the applied cytostatic regimens, and eventually to adapt treatment protocols in patients with MRD. Sensitive MRD techniques might also be useful to determine the optimal time point for harvesting BM grafts for autologous BM transplantation and to screen the obtained autologous grafts for the presence of residual leukemic cells. Whether adaptation of treatment protocols according to MRD data results in lower relapse rates and higher survival rates, should be investigated in prospective multicenter studies.

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SUMMARY

Acute myeloid leukemia (AML) is a malignancy characterized by an uncontrolled proliferation of immature myeloid cells in the bone marrow (BM). In AML there is a marked genotypic and phenotypic heterogeneity, which is reflected in a marked patient to patient variation in clinical presentation, response to therapy, and prognosis. In contrast to most patients with acute lymphoblastic leukemia (ALL), in the majority of AML patients also a marked intraclonal variation is present. To increase insight in this heterogeneity of AML, we applied extensive immunological marker analyses to characterize the various leukemic subpopulations which can be found in most AML patients. The results were correlated with other phenotypic, genotypic, and clinical features observed in these patients. The main objective of this study was to recognize specific subgroups of AML patients and to characterize immature subpopulations of AML cells which might be useful for monitoring the disease during follow-up.

Chapter 1 summarizes the present knowledge of the processes involved in normal hematopoiesis and leukemogenesis of AML. Especially literature data on epidemiology, biology, diagnosis, and treatment of this type of leukemia are summarized. AML is a clonal disorder, which occurs at all ages with an overall incidence in the Netherlands of 2.4 per 100,000 inhabitants per year. In most cases the etiology is unknown, although occupational and environmental exposures, such as radiation, benzene, and alkylating drugs are probably important in some AML cases. Most probably, alterations in the structure of critical genes, such as oncogenes, growth factor genes, and growth factor receptor genes are important in leukemogenesis. The occurrence of non-random chromosome aberrations related to specific biological and clinical characteristics supports the important pathogenetic role of genetic alterations.

Traditionally, AML is diagnosed and classified according to the French American British (FAB) classification system, which is based on cytomorphology and cytochemistry. Despite some correlations with biological features of AML, the prognostic value of the FAB classification is limited. Application of more advanced techniques, such as immunological marker analysis, cytogenetic analysis, and molecular analysis has enabled a further characterization of AML and recognition of various AML subtypes. Chemotherapy is the primary treatment option for AML patients. In addition, BM transplantation and more recently growth factors, differentiation factors, and immunotherapy are applied in some AML patients. Although most AML patients obtain complete remission (CR) (i.e. no tumor cells detectable by use of cytomorphology), the majority of patients will subsequently relapse and die of their disease. This indicates that leukemic cells persist despite cytomorphological CR, which emphasizes the importance of methods to detect low frequencies of leukemic cells to determine the effectiveness of the applied treatment.

Chapter 2 describes the various immunological markers and techniques for marker analysis. Most immunological markers represent differentiation antigens, which are well

characterized. Data concerning chromosome localization of the encoding genes, molecular mass and function of the antigens as well as (monoclonal) antibodies which recognize the antigens are summarized. One of the most characteristic markers of AML is CD33, a pan-myeloid differentiation antigen. In interlineage human-mouse hybrids, which were constructed by fusion of leukemic cells from an AML-M0 patient with a murine cell line we could demonstrate that expression of the pan-myeloid antigen CD33 depends on the presence of human chromosome 19 (Chapter 2.3).

Immunofluorescence (IF) methods are extremely useful for immunological marker analysis of hematopoietic cells, because they allow the simultaneous evaluation of two or three markers together with "morphological" parameters. Several aspects of IF microscopy and flow cytometry as well as the possibilities and limitations of both techniques are described in Chapters 2.1 and 2.2. It is obvious that for the interpretation of immunological marker analysis in AML one should have insight into the expression of these markers during normal and abnormal hematopoiesis. Information concerning the expression patterns of the various markers is summarized in the Chapters 2.1 and 2.2. Markers which have proven to be of value in AML diagnosis are discussed in more detail in Chapter 2.2.

One of the aims of our study was to recognize subgroups of AML which exhibit a specific phenotype. Since non-random chromosome aberrations are highly correlated with certain biological features of AML, we investigated whether karyotypic abnormalities are associated with a specific immunophenotype. In Chapter 3.1 common recurring chromosome abnormalities and their associations with specific AML features are summarized. Except for the unique $CD9^+$, $CD13^+$, $CD15^-$, $CD33^+$, $HLA\text{-}DR^-$ immunophenotype in AML-M3 with t(15;17)(q22;q21) and the $CD19^+$ immunophenotype in AML with t(8;21)(q22;q22), such associations are rare.

In two patients having AML-M4 with t(6;9)(p23;q34) we could demonstrate that the leukemic cells were $HLA\text{-}DR^+$, $CD13^+$, partly terminal deoxynucleotidyl transferase $(TdT)^+$ (Chapter 3.2). The CD33 antigen was expressed in one patient but negative in the other patient. In the former patient we analysed BM samples during follow-up for the presence of $CD33^+$, TdT^+ cells. We observed a gradual increase of $CD33^+$, TdT^+ cells in a period of 6 months before hematological relapse, which indicated that double IF staining for a myeloid marker and TdT might be useful for detection of minimal disease.

Chapter 3.3 describes our extensive immunophenotyping studies with double IF stainings to characterize the various subpopulations in eight patients having AML-M4Eo with inv(16)(p13q22). Despite heterogeneity within each patient, the immunophenotype of the subpopulations was strikingly similar. Virtually all AML-M4Eo cells were $CD13^+$, whereas several other markers, including CD2, CD11b, CD11c, CD14, CD33, CD34, CD36, CDw65, TdT, and HLA-DR were expressed by a part of the leukemic cells. Expression of the CD2 antigen was found within the immature ($CD34^+$) AML subpopulations as well as within the more mature ($CD14^+$) AML subpopulations, whereas TdT expression was exclusively found in the $CD34^+$, $CD14^-$ subpopulations. Because in T cells the CD2 antigen is known to function as an activation molecule, we cultured three AML-M4Eo samples. A high spontaneous proliferation was seen in all three patients. Addition of CD2 antibodies diminished cell proliferation in two patients with high CD2 expression, but no inhibitory effect was found in the third patient with

low frequency and low density of CD2 expression. These results suggest that expression of the CD2 molecule is involved in the proliferation of $CD2^+$ AML-M4Eo cells.

The finding of myeloid $marker^+$, TdT^+ cells in AML patients with t(6;9) led us to investigate whether these double positive cells could be used as a clue for the detection of minimal residual disease (MRD) (Chapter 4). First, it was determined whether $CD13^+$, TdT^+ and $CD33^+$, TdT^+ cells occurred in normal BM and peripheral blood (PB) samples as well as in cell samples taken from ALL patients in CR during and after chemotherapy (Chapter 4.1). The latter group of controls was included to investigate the effects of cytostatic treatment and BM regeneration on the occurrence of myeloid $marker^+$, TdT^+ cells. The results demonstrated that normal myeloid $marker^+$, TdT^+ cells are rare in BM (<0.03%, if they occur at all) and that such cells are not detectable in PB (Chapter 4.1).

In the study of Chapter 4.2 the occurrence of myeloid $marker^+$, TdT^+ cells in various subtypes of AML was investigated. In a series of 60 non-selected AML patients we found myeloid $marker^+$, TdT^+ cells in 45 patients (=75%). In contrast to TdT expression in ALL patients, TdT positivity in AML was often restricted to a subpopulation of the leukemic cells, which in most cases constituted less than 10% of the AML cells. Based on the relatively high expression of the CD34 antigen by the TdT^+ subpopulation as well as the proportional increase of myeloid $marker^+$, TdT^+ cells in relapsed patients, it was suggested that the TdT^+ cells represent an immature AML subpopulation, which might contain clonogenic AML cells. Our first results on the use of myeloid marker, TdT double IF staining for detection of MRD in AML patients are given in Chapters 4.1 and 4.2. In these patients a gradual increase of myeloid $marker^+$, TdT^+ cells was seen during a 3-6 months period before morphological relapse.

Chapter 4.3 summarizes the results of a prospective follow-up study in 14 additional patients with a TdT^+ AML. Twelve patients had obtained CR at the end of the chemotherapy courses. During subsequent follow-up seven out of these 12 patients developed one or two relapses (total of ten relapses). Nine out of these ten relapses were preceded by a gradual increase of myeloid $marker^+$, TdT^+ cells in BM and PB over a period of 14-38 weeks, indicating that the double IF staining method is a powerful tool to detect MRD in TdT^+ AML patients. The false negative result in one patient was caused by a phenotypic shift of the AML cells towards TdT negativity. In the five AML patients who were in continuous CR relatively high percentages of myeloid $marker^+$, TdT^+ cells were repeatedly detected in BM (up to 0.1%) and PB (up to 0.02%). Although the leukemic origin of these double positive cells could not be proven, we speculate that these cells represent residual dysplastic AML cells which survived chemotherapy but which were not yet capable of causing leukemic regrowth.

In precursor lymphoid cells TdT is expressed and functionally active during rearrangements of immunoglobulin (Ig) and T cell receptor (TcR) genes resulting in extra nucleotide insertion at the junctional regions of these rearranging genes. In the study of Chapter 4.4 we investigated whether the expression of TdT in AML was related to the occurrence of cross-lineage Ig and/or TcR gene rearrangements. In contrast to other reports, we investigated all Ig and TcR gene complexes, i.e. IgH, Igκ, Igλ, TcR-β, TcR-γ, and TcR-δ. In 15% (8/54) of the AML one or more genes were clonally rearranged. This especially concerned IgH genes. No relationship between the occurrence of cross-lineage rearrangements and TdT positivity was

found. Analysis of our results and those of over 400 cases published in the literature demonstrated a high concurrence of cross-lineage rearrangements in AML. This finding supports the hypothesis of a common recombinase for Ig and TcR genes. In addition, the results suggested that cross-lineage rearrangements probably occur in a non-randomly ordered fashion, in which rearrangements of IgH and TcR-δ genes precede the other gene rearrangements.

In Chapter 5 the results of our AML studies are discussed in more detail. Furthermore, some recently obtained data are presented and discussed in the context of the latest data from the literature. Preliminary results of recently performed experiments using a monoclonal antibody against the c-kit antigen revealed that this antigen is expressed on a part of the immature $CD34^+$ AML cells. In addition, we could demonstrate coexpression of c-kit and TdT in TdT^+ AML, which supports our hypothesis that TdT^+ subpopulations represent immature AML cells. Furthermore, these results suggest that c-kit might be an useful marker to apply in double IF stainings for the detection of MRD.

The detection of MRD is a major problem in AML patients. In Chapter 5 several methods for MRD detection are described and compared. From this comparison it can be concluded that multiparameter analysis, especially myeloid marker, TdT double IF stainings and PCR techniques give the best results.

We conclude that multiparameter immunological marker analysis is a powerful tool to phenotype the various subpopulations in heterogeneous disorders like AML. These studies give insight into the differential maturation arrest of the various types of AML. The recognition and characterization of immature subpopulations within each AML clone is crucial for the development of methods for MRD detection. Application of methods for MRD detection in AML patients during follow-up can be used to adjust remission and relapse criteria, to monitor the effectiveness of the applied cytostatic regimens, and finally to adapt treatment protocols in patients with MRD. Whether adaptation of treatment protocols according to MRD data results in lower relapse rates and higher survival rates should be investigated in prospective multicenter studies.

SAMENVATTING

Acute myeloïde leukemie (AML) is een vorm van bloedkanker die wordt gekarakteriseerd door een ongecontroleerde proliferatie van onrijpe myeloïde cellen in het beenmerg. Tussen de patiënten met AML bestaat een duidelijke genotypische en fenotypische heterogeniteit, die zich ondermeer uit in een grote variatie in klinische presentatie, therapierespons en prognose. In tegenstelling tot de meeste patiënten met acute lymfatische leukemie (ALL), wordt bij vrijwel alle patiënten met een AML tevens een duidelijke intraklonale heterogeniteit gezien. Om het inzicht in de heterogeniteit van AML te vergroten en om de verschillende subpopulaties te karakteriseren die aanwezig zijn bij de meeste AML patiënten, hebben wij uitgebreide immunologische markeranalyses uitgevoerd. De resultaten werden gecorreleerd met andere fenotypische, genotypische en klinische bevindingen bij deze patiënten. Het primaire doel van deze studie was om specifieke subgroepen van AML patiënten te herkennen en om onrijpe leukemische subpopulaties te karakteriseren, die gebruikt kunnen worden om het verloop van de ziekte en de effektiviteit van de behandeling te evalueren.

Hoofdstuk 1 vat informatie samen over de processen die een rol spelen bij de normale hematopoïese en bij het ontstaan van AML. In het bijzonder worden de epidemiologie, biologie, diagnose en behandeling van deze vorm van leukemie besproken. AML is een klonale aandoening met in Nederland een incidentie van 2,4 nieuwe patiënten per 100.000 inwoners per jaar. In de meeste gevallen is de etiologie onbekend, maar blootstelling aan straling, benzeen en alkylerende chemotherapeutica speelt waarschijnlijk een rol bij een aantal patiënten. Veranderingen in bepaalde genen, zoals oncogenen, groeifaktor genen en groeifaktorreceptor genen, zijn waarschijnlijk van groot belang voor het ontstaan van AML. Het pathogenetisch belang van genetische veranderingen wordt onderstreept door de associatie tussen "non-random" chromosoom veranderingen en specifieke biologische en klinische kenmerken van AML.

AML wordt traditioneel gediagnostiseerd en geklassificeerd volgens het Frans-Amerikaans-Britse (FAB) klassificatiesysteem, dat gebaseerd is op cytomorfologische en cytochemische kenmerken van de leukemiecellen. Ondanks enkele correlaties met biologische kenmerken van AML, is de prognostische waarde van de FAB klassificatie beperkt. Door toepassing van meer geavanceerde technieken, zoals immunologische markeranalyse, cytogenetische analyse en moleculaire analyse, is nadere karakterisering van AML mogelijk en kunnen verschillende AML subtypen worden onderscheiden. Patiënten met AML worden in het algemeen behandeld door middel van chemotherapie. Daarnaast bestaat er voor bepaalde patiënten de mogelijkheid tot beenmerg transplantatie en, sinds kort, de toepassing van groei- en/of differentiatiefaktoren en immunotherapie. Ondanks het feit dat de meeste patiënten met AML een complete remissie (CR) bereiken (er kunnen dan cytomorfologisch geen leukemiecellen meer worden aangetoond), krijgen veel patiënten uiteindelijk een recidief en sterven zij aan de leukemie. Dit betekent dat, ondanks de behandeling en de schijnbare CR, toch AML cellen aanwezig blijven,

hetgeen het grote belang benadrukt van het ontwikkelen van methoden om kleine aantallen leukemiecellen te detecteren. Dergelijke methoden zouden kunnen worden gebruikt om nauwkeurig de effektiviteit van de ingestelde therapie te bepalen.

In hoofdstuk 2 wordt gedetailleerde informatie gegeven over de verschillende immunologische markers en de technieken die gebruikt kunnen worden voor immunologische markeranalyse. De meeste immunologische markers zijn differentiatie antigenen en inmiddels goed gekarakteriseerd. Kennis betreffende de chromosoomlokalisatie van de coderende genen, het molekuulgewicht en de funktie van deze antigenen, en van de (monoklonale) antistoffen die deze antigenen specifiek herkennen, is samengevat in de hoofdstukken 2.1 en 2.2. Eén van de meest kenmerkende markers voor AML is CD33, een myeloïd differentiatie antigeen. In mens-muis hybride cellen, die gemaakt werden door fusie van leukemiecellen van een patiënt met AML-M0 met een muizecellijn, konden wij aantonen dat de expressie van CD33 afhankelijk is van de aanwezigheid van het menselijke chromosoom 19 (hoofdstuk 2.3).

Immunofluorescentie (IF)' methoden zijn uitermate geschikt voor het uitvoeren van immunologische markeranalyse, omdat zij gelijktijdige analyse van twee of drie markers tezamen met "morfologische" parameters mogelijk maken. Verschillende aspecten van IF microscopie en flowcytometrie worden respectievelijk besproken in hoofdstukken 2.1 en 2.2. In deze hoofdstukken vergelijken wij de mogelijkheden en beperkingen van beide technieken. Voor een juiste interpretatie van immunologische markeranalyse van AML is kennis van de expressiepatronen van de verschillende antigenen tijdens normale en abnormale hematopoïese belangrijk. In hoofdstuk 2.2 wordt het diagnostisch belang van verschillende markers bij patiënten met AML besproken.

Een van de doelen van ons onderzoek was om subgroepen van AML patiënten te kunnen onderscheiden op basis van een specifiek immunofenotype. Gezien de sterke associatie tussen bepaalde "non-random" chromosoomafwijkingen en biologische kenmerken van AML, onderzochten wij of bij AML associaties bestaan tussen chromosoom afwijkingen en specifieke immunofenotypen. In hoofdstuk 3.1 zijn de meest frequente chromosoomafwijkingen samengevat die worden gevonden bij AML patiënten alsmede hun associaties met bepaalde kenmerken van de leukemie. Met uitzondering van het unieke $CD9^+$, $CD13^+$, $CD15^-$, $CD33^+$, $HLA\text{-}DR^-$ immunofenotype bij AML-M3 met t(15;17)(q22;q21) en het $CD19^+$ immunofenotype bij AML met t(8;21)(q22;q22), zijn geen duidelijke associaties bekend.

Bij twee patiënten met een AML-M4 met t(6;9)(p23;q34) bleken de leukemiecellen positief te zijn voor HLA-DR en CD13 en gedeeltelijk positief voor terminaal deoxynucleotidyl transferase (TdT) (hoofdstuk 3.2). Het CD33 antigeen was aantoonbaar bij één van de twee patiënten. Tijdens de follow-up van deze patiënt werden beenmerg monsters onderzocht op de aanwezigheid van $CD33^+$, TdT^+ cellen. Gedurende een periode van 6 maanden voordat de patiënt een morfologisch bewezen recidief kreeg, werd een geleidelijke toename van het percentage $CD33^+$, TdT^+ cellen aangetoond. Deze bevinding toont aan dat de dubbel IF kleuring voor een myeloïde marker en TdT nuttig kan zijn voor het opsporen van kleine aantallen AML cellen.

Hoofdstuk 3.3 beschrijft de resultaten van uitgebreide immunologische markeranalyses met dubbel IF kleuringen om de verschillende subpopulaties te karakteriseren bij acht patiënten met

een AML-M4Eo met inv(16)(p13q22). Ondanks de heterogeniteit bij elke patiënt bleek het immunofenotype van de verschillende subpopulaties bij deze acht patiënten sterk overeen te komen. Vrijwel alle AML-M4Eo cellen waren $CD13^+$, terwijl andere immunologische markers, zoals CD2, CD11b, CD11c, CD14, CD33, CD34, CD36, CDw65, TdT en HLA-DR, door een deel van leukemiecellen tot expressie werden gebracht. Expressie van het CD2 antigeen kon worden aangetoond binnen zowel de onrijpe ($CD34^+$) AML subpopulaties als de rijpere ($CD14^+$) AML subpopulaties, terwijl de expressie van TdT beperkt bleef tot de $CD34^+$, $CD14^-$ subpopulaties. Omdat het CD2 antigeen bij T-cellen functioneert als een aktivatiemolekuul, hebben we drie AML-M4Eo celmonsters in kweek gebracht. In de celkweken van deze drie patiënten werd een hoge spontane proliferatie gemeten. Toediening van CD2 antistoffen resulteerde in een duidelijke vermindering van de proliferatie bij twee van de drie patiënten. Bij de derde patiënt werd geen inhibitie van de proliferatie aangetoond, hetgeen waarschijnlijk samenhing met het lage percentage $CD2^+$ cellen en de geringe mate van CD2 expressie per cel. Deze resultaten suggereren dat expressie van het CD2 molekuul een rol speelt bij de proliferatie van de $CD2^+$ AML-M4Eo cellen.

De aanwezigheid van myeloïde $marker^+$, TdT^+ cellen tijdens en na behandeling van de leukemie, in AML patiënten met een t(6;9) stimuleerde ons om te onderzoeken of op basis van deze markers kleine aantallen AML cellen konden worden aangetoond (hoofdstuk 4). Eerst werd bepaald of $CD13^+$, TdT^+ cellen en $CD33^+$, TdT^+ voorkomen in normale beenmerg- en bloedcelmonsters en in celmonsters van patiënten met een ALL in CR tijdens en na het stoppen van de chemotherapie (hoofdstuk 4.1). De ALL patiëntengroep werd onderzocht om te bepalen of cytostatische behandeling en beenmerg regeneratie van invloed zijn op het voorkomen van myeloïde $marker^+$, TdT^+ cellen. De resultaten toonden aan dat myeloïde $marker^+$, TdT^+ cellen soms in lage percentages in beenmerg aanwezig zijn (<0,03%) en dat zulke cellen normaliter niet in het bloed detecteerbaar zijn (hoofdstuk 4.1).

In hoofdstuk 4.2 wordt het vòòrkomen van myeloïde $marker^+$, TdT^+ cellen in verschillende subtypen van AML beschreven. In een groep van 60 ongeselecteerde AML patiënten werden bij 45 patiënten (=75%) myeloïde $marker^+$, TdT^+ cellen aangetoond. In tegenstelling tot de expressie van TdT in patiënten met een ALL, bleek de TdT positiviteit bij patiënten met een AML meestal beperkt te zijn tot een leukemische subpopulatie, die bij het merendeel van de patiënten <10% van de AML cellen bleek te beslaan. Omdat binnen de TdT^+ subpopulatie een relatief hoge expressie van het CD34 antigeen werd gevonden, en er bij het recidief een toename van de myeloïde $marker^+$, TdT^+ celpopulatie werd gezien, hebben wij gepostuleerd dat de TdT^+ AML cellen een onrijpe, mogelijk klonogene, leukemische celpopulatie vertegenwoordigen. De eerste resultaten van het gebruik van myeloïde marker, TdT dubbelkleuringen voor het opsporen van kleine aantallen tumorcellen bij patiënten onder en na AML behandeling zijn beschreven in de hoofdstukken 4.1 en 4.2. In de onderzochte patiënten kon een geleidelijke toename van myeloïde $marker^+$, TdT^+ cellen worden aangetoond 3 tot 6 maanden vóór het ontstaan van een cytomorfologisch recidief.

In hoofdstuk 4.3 worden de resultaten beschreven van een prospectief vervolgonderzoek van 14 patiënten met een TdT^+ AML. Twaalf patiënten bleken na hun laatste chemotherapiekuur in CR te zijn. Vervolgens kregen zeven van deze 12 patiënten één of twee recidieven

(totaal tien recidieven). Negen van deze tien recidieven werden voorafgegaan door een geleidelijke toename van myeloïde marker$^+$, TdT$^+$ cellen in beenmerg en bloed gedurende een periode van 14 tot 38 weken. Deze resultaten tonen aan dat de dubbel IF kleuring een geschikte methode is voor het opsporen van kleine aantallen leukemiecellen bij patiënten met een TdT$^+$ AML. Het vals-negatieve resultaat bij één patiënt werd veroorzaakt door verlies van TdT expressie door de AML cellen bij het recidief. Bij de vijf patiënten, die in eerste continue CR waren, werden regelmatig relatief hoge percentages myeloïde marker$^+$, TdT$^+$ cellen aangetoond in het beenmerg (<0,1%) en in het bloed (<0,02%). Hoewel niet kon worden bewezen dat deze dubbelpositieve cellen leukemisch waren, is het zeer goed mogelijk dat deze cellen resterende dysplastische AML cellen zijn die de chemotherapie overleefden, maar die (nog) niet in staat waren om een recidief van de leukemie te veroorzaken.

In voorloper lymfatische cellen speelt het enzym TdT een belangrijke rol tijdens de herschikkingen van de immunoglobuline (Ig) en T-cel receptor (TcR) genen, doordat het extra nucleotiden invoegt op de verbindingsplaatsen van de herschikkende genen. In hoofdstuk 4.4 werd onderzocht of TdT expressie in AML samenhangt met het plaatsvinden van zogenaamde "cross-lineage" herschikkingen van Ig en/of TcR genen. In tegenstelling tot eerdere studies van andere onderzoekers, onderzochten wij de genconfiguratie van alle bekende Ig en TcR gencomplexen, te weten IgH, Igκ, Igλ, TcR-β, TcR-γ en TcR-δ. In 15% (8/54) van de AML patiënten bleek dat één of meerdere genen klonaal herschikt waren. Dit betrof voornamelijk IgH genherschikkingen. Er was geen correlatie aantoonbaar tussen de aanwezigheid van deze "cross-lineage" herschikkingen en de expressie van TdT. Analyse van onze resultaten en de gepubliceerde resultaten van meer dan 400 andere patiënten liet zien dat "cross-lineage" herschikkingen van verschillende genen vaak samen voorkomen. Deze bevinding steunt de hypothese dat er één "recombinase" enzymsysteem bestaat voor zowel Ig- als TcR genherschikkingen. Daarnaast bleek dat de herschikkingen van de verschillende genen waarschijnlijk in een vaste volgorde verlopen, waarbij herschikkingen van de IgH en TcR-δ genen aan de andere genherschikkingen voorafgaan.

In Hoofdstuk 5 worden de resultaten van het AML onderzoek nader bediscussieerd. Tevens worden resultaten van enkele recente experimenten besproken. Zo werd met een anti-c-kit antistof aangetoond dat c-kit tot expressie komt op een deel van de onrijpe CD34$^+$ AML cellen. Daarnaast konden wij coëxpressie van c-kit en TdT aantonen bij patiënten met een TdT$^+$ AML, hetgeen onze hypothese steunt dat de TdT$^+$ subpopulatie inderdaad onrijpe AML cellen bevat. Verder suggereren deze resultaten dat c-kit mogelijk een nuttige marker kan zijn bij dubbel IF kleuringen voor de detectie van kleine aantallen AML cellen.

De detectie van kleine aantallen leukemiecellen is een belangrijk probleem bij de behandeling van patiënten met een AML. In hoofdstuk 5 worden verschillende methoden voor het opsporen van kleine aantallen AML cellen besproken en vergeleken. De beste resultaten worden thans verkregen met multiparameter analyse (in het bijzonder de myeloïde marker, TdT dubbelkleuring) en PCR technieken.

Wij concluderen dat multiparameter immunologische markeranalyse een geschikte techniek is voor het immunofenotyperen van de verschillende subpopulaties bij heterogene aandoeningen zoals AML. Zulke analyses geven inzicht in de mate van maturatie-arrest van de

verschillende AML typen. Het herkennen en karakteriseren van onrijpe AML subpopulaties is noodzakelijk voor het ontwikkelen van methoden om kleine aantallen leukemiecellen op te sporen. Toepassing van dergelijke methoden bij het vervolgen van patiënten met AML tijdens en na behandeling zou kunnen leiden tot het aanpassen van de criteria voor remissie en recidief, tot het verkrijgen van meer inzicht in de effektiviteit van de verschillende cytostaticakuren en eventueel tot het aanpassen van het behandelingsschema van patiënten met residuele AML cellen. Om te bepalen of het aanpassen van behandelingsschema's op geleide van genoemde technieken uiteindelijk zal leiden tot een lager aantal recidieven en een betere overleving, dienen prospectieve "multicenter" studies te worden verricht.

ABBREVIATIONS

AA : aplastic anemia
ABMT : autologous bone marrow transplantation
AET : 2-amino-ethylisothiouronium bromide
AIDS : acquired immunodeficiency syndrome
ALL : acute lymphoblastic leukemia
AML : acute myeloid leukemia
ANLL : acute non-lymphoblastic leukemia
AO : acridine orange
AP : alkaline phosphatase
Ara-C : cytosine arabinoside
AUL : acute undifferentiated leukemia
B-ALL : B cell acute lymphoblastic leukemia
B-CLL : B cell chronic lymphocytic leukemia
B-PLL : B cell prolymphocytic leukemia
BAL : bronchoalveolar lavage
BC : blast crisis
BFU-E : erythrocyte burst forming unit
BM : bone marrow
BMT : bone marrow transplantation
BSA : bovine serum albumin
CALLA : common acute lymphoblastic leukemia antigen
CD : cluster of differentiation/cluster of designation
CDw : CD "workshop" (preliminary clustering)
CFU : colony forming unit
CLL : chronic lymphocytic leukemia
CML : chronic myeloid leukemia
CNS : central nervous system
CR : complete remission
CSF : cerebrospinal fluid; colony stimulating factor
CyCD3 : cytoplasmic expression of CD3 antigen
CyIg : cytoplasmic immunoglobulin
Cyμ : cytoplasmic μ heavy chain
D : diversity
DIC : disseminated intravascular coagulation
DNA : deoxyribonucleic acid
EM : electron microscopy
Epo : erythropoietin
F : female
FAB : French American British cytomorphological classification of acute leukemias
FcγR : Fc receptor for IgG
FCS : fetal calf serum
FITC : fluorescein isothiocyanate
FISH : fluorescent in situ hybridization
FSC : forward scatter
G-CSF : granulocyte colony stimulating factor
GM-CSF : granulocyte macrophage colony stimulating factor
GP or gp : glycoprotein
GpA : glycophorin A
GVH : graft versus host
Hb : hemoglobin
HCL : hairy cell leukemia

HPRT : hypoxanthine phosphoribosyl transferase
IF : immunofluorescence
Ig : immunoglobulin
IgH : immunoglobulin heavy chain
IL : interleukin
Inr : initiator
J : joining
kb : kilobase
kDa : kilo Dalton
LFA : leukocyte function antigen
M : male
McAb : monoclonal antibody
M-CSF : macrophage colony stimulating factor
MDR : multidrug resistance
MDS : myeloid dysplastic syndrome
M4Eo : AML-M4 with bone marrow eosinophilia
MIC : morphologic, immunologic, and cytogenetic classification
MNC : mononuclear cells
MPO : myeloperoxidase
MRBC : mouse red blood cells
MRD : minimal residual disease
NCAM : neural cell adhesion molecule
ND : not done
NHL : non-Hodgkin lymphoma
NK cell : natural killer cell
NMS : normal mouse serum
NT : not tested
PB : peripheral blood
PBS : phosphate buffered saline
PCR : polymerase chain reaction
PE : phycoerythrin
PI : phosphatidylinositol glycan
PKC : protein kinase C
PLL : prolymphocytic leukemia
PMA : phorbol 12-myristate 13-acetate
PNH : paroxysmal nocturnal hemoglobinuria
PO : peroxidase
PR : partial remission
RA : retinoic acid
RAR : retinoic acid receptor
RT : room temperature
SCF : stem cell factor
SmIg : surface membrane immunoglobulin
SRBC : sheep red blood cells
SSC : sideward scatter
T-ALL : T cell acute lymphoblastic leukemia
T-NHL : T cel non-Hodgkin lymphoma
TcR : T cell receptor
TdT : terminal deoxynucleotidyl transferase
TGF : transforming growth factor
TNF : tumor necrosis factor
TRITC : tetramethylrhodamine isothiocyanate
V : variable
WBC : white blood cells

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