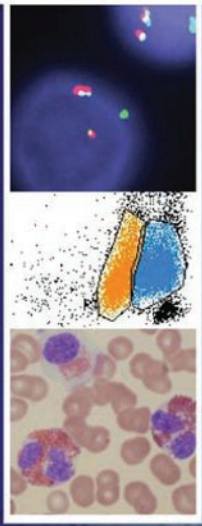
Barbara J. Bain

Fourth Edition

Leukaemia Diagnosis



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LEUKAEMIA DIAGNOSIS

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PREFACE

Leukaemias are a very heterogeneous group of diseases, which differ from each other in aetiology, pathogenesis, prognosis and responsiveness to treatment. Accurate diagnosis and classification are necessary for the identification of specific biological entities and underpin scientific advances in this field. The detailed characterization of haematological neoplasms is also essential for the optimal management of individual patients. Many systems for the classification of leukaemia have been proposed. Between 1976 and 1999, a collaborative group of French, American and British haematologists (the FAB group) proposed a number of classifications, which became widely accepted throughout the world. In 2001, a quarter of a century after the first FAB proposals, a World Health Organization (WHO) expert group proposed an updated system for the classification of leukaemia and lymphoma incorporating clinical features, haematological and histological features, immunophenotyping and the results of cytogenetic and, to a lesser extent, molecular genetic analysis. In 2008 a further updating of the WHO classification incorporated new knowledge and gave a greater importance to molecular genetic features.

In this book I have sought to illustrate and explain how these many laboratory techniques are used for the diagnosis and classification of leukaemia and related disorders. I have sought to discuss diagnosis and classification in a way that will be helpful to trainee haematologists and to laboratory scientists in haematology and related disciplines. However, I have also tried to provide a useful reference source and teaching aid for those who already have expertise in this field. In addition, I hope that cytogeneticists and molecular geneticists will find that this book enhances their understanding of the relationship of their disciplines to the diagnosis, classification and monitoring of leukaemia and related disorders.

As the diagnosis and classification of leukaemia comes to rely increasingly on sophisticated and expensive investigations there is a risk that some countries will be left behind. For the first time I have therefore incorporated, as an appendix, some suggestions as to how leukaemia might be diagnosed in under-resourced laboratories. This may seem presumptuous from someone who has not worked in a developing country for more than 40 years but I thought that at least it should be attempted.

Since photographs have been taken of many different microscopes and exact magnifications differ slightly, magnifications of photomicrographs in this edition are given as the microscope objective used.

Acknowledgements

I should like to record my gratitude to two founder members of the FAB group, the late Professor David Galton and Professor Daniel Catovsky, both of whom gave me a great deal of help in developing this book but at the same time left me free to express my own opinions. Professor Galton read the entire manuscript of the first edition and, by debating many difficult points with me, gave me the benefit of his wisdom and experience. Professor Catovsky also discussed problem areas and kindly permitted me to photograph blood and bone marrow films from many of his patients. Others helped by reading part or all of the manuscripts of subsequent editions. My thanks are also due to the considerable number of friends and colleagues from four continents who helped by lending material for photography or supplying photographs; they are individually acknowledged in the relevant figure legends.

Barbara J. Bain 2009

ABBREVIATIONS

Specific cytogenetic abbreviations are shown in Table 2.13.

aCML	atypical chronic myeloid leukaemia (category in WHO classification)
AIDS	acquired immune deficiency syndrome
AIHA	autoimmune haemolytic anaemia
ALIP	abnormal localization of immature precursors
ALL	acute lymphoblastic leukaemia
AML	acute myeloid leukaemia
ANAE	α-naphthyl acetate esterase (cytochemical stain)
ANBE	α-naphthyl butyrate esterase (cytochemical stain)
ATLL	adult T-cell leukaemia/lymphoma
ATRA	all-*trans*-retinoic acid
BCSH	British Committee for Standards in Haematology
BFU-E	burst-forming unit – erythroid
BM	bone marrow
c	cytoplasmic or, in cytogenetic terminology, constitutional
CAE	naphthol AS-D chloroacetate esterase, chloroacetate esterase (cytochemical stain)
CBF	core binding factor
CD	cluster of differentiation
CEL	chronic eosinophilic leukaemia
CFU-E	colony-forming unit – erythroid
CFU-GM	colony-forming unit – granulocyte, macrophage
CFU-Meg	colony-forming unit – megakaryocyte
CGH	comparative genomic hybridization
CGL	chronic granulocytic leukaemia
cIg	cytoplasmic immunoglobulin
CLL	chronic lymphocytic leukaemia
CLL/PL	chronic lymphocytic leukaemia, mixed cell type with prolymphocytoid cells
CMML	chronic myelomonocytic leukaemia (category in FAB and WHO classifications)
CT	computerized tomography
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
EBV	Epstein–Barr virus
EDTA	ethylene diamine tetra-acetic acid
EGIL	European Group for the Immunological Characterization of Leukemias
ERFC	E-rosette-forming cells
FAB	French–American–British (classification)
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate
FSC	forward light scatter (flow cytometry term)
G-CSF	granulocyte colony-stimulating factor
GM-CSF	granulocyte–macrophage colony-stimulating factor
H&E	haematoxylin and eosin (stain)
Hb	haemoglobin concentration
HCL	hairy cell leukaemia
HIV	human immunodeficiency virus
HLA-DR	human leucocyte antigen DR
HTLV-I	human T-cell lymphotrophic virus I
Ig	immunoglobulin
IGH	immunoglobulin heavy chain (locus)
IGVH	immunoglobulin heavy chain variable region (genes)
IPSS	International Prognostice Scoring System
ITD	internal tandem duplication

JMML	juvenile myelomonocytic leukaemia (category in WHO classification)
LDH	lactate dehydrogenase
LGL	large granular lymphocyte/s
M : E	myeloid : erythroid (ratio)
M0–M7	categories of acute myeloid leukaemia in the FAB classification
MAC	morphology–antibody–chromosomes (technique)
MALT	mucosa-associated lymphoid tissue
m-BCR	minor breakpoint cluster region
M-BCR	major breakpoint cluster region
McAb	monoclonal antibody/ies
MDS	myelodysplastic syndrome/s
MDS/MPN	myelodysplastic/myeloproliferative neoplasm/s
MDS-U	myelodysplastic syndrome, unclassifiable (category in WHO classification)
MGG	May–Grünwald–Giemsa (a stain)
MIC	Morphology, Immunophenotype, Cytogenetics (classification)
MIC-M	Morphology, Immunophenotype, Cytogenetics, Molecular genetics (classification)
MPAL	mixed phenotype acute leukaemia
MPN	myeloproliferative neoplasm/s
MPO	myeloperoxidase (cytochemical stain)
MRC	Medical Research Council
MRD	minimal residual disease
mRNA	messenger RNA
NAP	neutrophil alkaline phosphatase
NASA	naphthol AS acetate esterase (cytochemical stain)
NASDA	naphthol AS-D acetate esterase (cytochemical stain)
NHL	non-Hodgkin lymphoma/s
NK	natural killer
NOS	not otherwise specified
NSE	non-specific esterase (cytochemical stain)
PAS	periodic acid–Schiff (cytochemical stain)
PB	peripheral blood
PcAb	polyclonal antibody/ies
PCR	polymerase chain reaction
PE	phycoerythrin
Ph	Philadelphia (chromosome)
PLL	prolymphocytic leukaemia
PPO	platelet peroxidase
PTD	partial tandem duplication
RA	refractory anaemia (category in FAB and WHO classification)
RAEB	refractory anaemia with excess of blasts (category in FAB classification)
RAEB-T	refractory anaemia with excess of blasts in transformation (category in FAB classification)
RARS	refractory anaemia with ring sideroblasts (category in FAB and WHO classifications)
RARS-T	refractory anaemia with ring sideroblasts and thrombocytosis (provisional category in WHO classification)
RC	refractory cytopenia (category in WHO classification)
RCC	refractory cytopenia of childhood (category in WHO classification)
RCMD	refractory cytopenia with multilineage dysplasia (category in WHO classification)
RCUD	refractory cytopenia with unilineage dysplasia
RN	refractory neutropenia (category in WHO classification)
RNA	ribonucleic acid
RQ-PCR	real-time quantitative polymerase chain reaction
RT	refractory thrombocytopenia (category in WHO classification)
RT-PCR	reverse transcriptase polymerase chain reaction
SBB	Sudan black B (cytochemical stain)
SLVL	splenic lymphoma with villous lymphocytes
SmIg	surface membrane immunoglobulin
SMZL	splenic marginal zone lymphoma
SSC	sideways light scatter (flow cytometry term)
TAM	transient abnormal myelopoiesis
t-AML	therapy-related acute myeloid leukaemia
TCR	T-cell receptor
TCR	T-cell receptor (locus)
TdT	terminal deoxynucleotidyl transferase

TKD	tyrosine kinase domain
t-MDS	therapy-related myelodysplastic syndrome
TRAP	tartrate-resistant acid phosphatase
WBC	white blood cell count
WHO	World Health Organization
WPSS	WHO-classification-based Prognostic Scoring System
ZAP70	zeta-associated protein 70

ONE

THE NATURE OF LEUKAEMIA, CYTOLOGY, CYTOCHEMISTRY AND THE FAB CLASSIFICATION OF ACUTE LEUKAEMIA

The nature of leukaemia

Leukaemia is a disease resulting from the neoplastic proliferation of haemopoietic or lymphoid cells. It results from mutation of a single stem cell, the progeny of which form a clone of leukaemic cells. Usually there is a series of genetic alterations rather than a single event. Genetic events contributing to malignant transformation include inappropriate expression of oncogenes and loss of function of tumour suppressor genes. Oncogenes may be normal cellular genes – proto-oncogenes, that have mutated or are dysregulated – or may be novel hybrid genes resulting from fusion of parts of two genes. The cell in which the leukaemic transformation occurs may be a lymphoid precursor, a myeloid precursor or a pluripotent haemopoietic stem cell

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capable of differentiating into both myeloid and lymphoid cells. Myeloid leukaemias can arise in a lineage-restricted cell, in a multipotent stem cell capable of differentiating into cells of erythroid, granulocytic, monocytic and megakaryocytic lineages or in a pluripotent lymphoid–myeloid stem cell.

Genetic alterations leading to leukaemic transformation often result from major alterations in the chromosomes, which can be detected by microscopic examination of the chromosomes of cells in metaphase. Other changes, such as point mutations or partial duplications, are at a submicroscopic level but can be recognized by analysis of deoxynucleic acid (DNA) or ribonucleic acid (RNA).

Neoplastic cells are genetically unstable so that further mutations can occur in cells of the clone. If a new mutation gives the progeny of that cell a growth or survival advantage it tends to replace the parent clone. Such clonal evolution can lead to transformation into a more aggressive or treatment-refractory form of the disease with an associated worsening of prognosis. A series of mutations can occur with progressive worsening of prognosis at each stage.

Leukaemias are broadly divided into: (i) acute leukaemias, which, if untreated, lead to death in weeks or months; and (ii) chronic leukaemias, which, if untreated, lead to death in months or years. They are further divided into lymphoid, myeloid and mixed lineage (biphenotypic or bilineage) leukaemias, the latter showing usually both lymphoid and myeloid differentiation. Acute leukaemias are characterized by a defect in maturation, leading to an imbalance between proliferation and maturation; since cells of the leukaemic clone continue to proliferate without maturing to end cells and dying there is continued expansion of the leukaemic clone and immature cells predominate. Chronic leukaemias are characterized by an expanded pool of proliferating cells that retain their capacity to differentiate to end cells.

The clinical manifestations of the leukaemias are due, directly or indirectly, to the proliferation of leukaemic cells and their infiltration into normal tissues. Increased cell proliferation has metabolic consequences and infiltrating cells also disturb tissue function. Anaemia, neutropenia and thrombocytopenia are important consequences of infiltration of the bone marrow, which in turn can lead to infection and haemorrhage.

The importance of classification

The purpose of any pathological classification is to bring together cases that have fundamental similarities and that are likely to share features of causation, pathogenesis and natural history. Making an accurate diagnosis of a haematological neoplasm is crucial for selection of the most appropriate treatment. Since there are many dozens, if not hundreds, of different types of leukaemia it is essential to have a classification that an individual case can be related to. Identification of homogeneous groups of biologically similar cases is important as it permits an improved understanding of the leukaemic process and ultimately benefits individual patients. Since such diagnostic categories or subgroups may differ from each other in the cell lineage affected, natural history, optimal choice of treatment and prognosis with and without treatment, their recognition permits the development of a selective evidence-based therapeutic approach with a resultant overall improvement in outcome. Identifying valid diagnostic categories also increases the likelihood of causative factors and pathogenetic mechanisms being recognized.

The development of the French–American–British (FAB) classification of acute leukaemia [1–5], and subsequently of other leukaemias and related conditions, by a collaborating group of French, American and British haematologists was a major advance in leukaemia classification, permitting uniform diagnosis and classification of these diseases over three decades. The FAB classification was based on morphology supplemented by cytochemistry and to some extent by immunophenotyping. Over the last decade the FAB classification has been increasingly supplemented or replaced by the *World Health Organization (WHO) Classification of Tumours of Haematopoietic and Lymphoid Tissues* [6]. The WHO classification is based on morphology (either cytology or histology) but also makes extensive use of immunophenotyping and of cytogenetic and molecular genetic analysis. The FAB classification continues to be of value for the preliminary morphological assessment of a case, since a careful

morphological assessment indicates which supplementary tests are indicated and provides a context in which such tests can be interpreted. The FAB classification also remains in use in circumstances where immunophenotypic and genetic analysis is not readily available and in this circumstance it is important that cytochemistry is not neglected. However, since a precise diagnosis is important for choice of treatment it is desirable that even resource-poor countries should try to establish those diagnostic methods that are essential for optimal patient management and outcome.

For clarity, it is important that FAB designations (which have a precise, carefully defined meaning) are not applied to WHO categories for which the diagnostic criteria differ. Publications, particularly those relating to acute leukaemia and the myelodysplastic syndromes (MDS), should state which classification is being used and should adhere strictly to the criteria of the relevant classification.

The nature and classification of acute leukaemia

Acute leukaemia comprises a heterogeneous group of conditions that differ in aetiology, pathogenesis, molecular mechanisms and prognosis. The heterogeneity is reduced if cases of acute leukaemia are divided into acute myeloid leukaemia (AML) (in North America previously designated 'acute nonlymphoblastic leukaemia'), acute lymphoblastic leukaemia (ALL) and mixed phenotype acute leukaemia (MPAL); even then, however, considerable heterogeneity remains within each of the groups.

Although the best criteria for categorizing a case of acute leukaemia as myeloid or lymphoid may be disputed, the importance of such categorization is beyond doubt. Not only does the natural history differ but the best current modes of treatment are still sufficiently different for an incorrect categorization to adversely affect prognosis. Assigning patients to subtypes of AML or ALL is becoming increasingly important as the benefits of more targeted treatment are identified. Similarly, the suspected poor prognosis of MPAL suggests that the identification of such cases may lead to a different therapeutic approach and an improved outcome. Cases of acute leukaemia can be classified on the basis of morphology, cytochemistry, immunophenotype, cytogenetic abnormality or molecular genetic abnormality, or by combinations of these characteristics. Morphology and cytochemistry of acute leukaemia will be discussed in this chapter, other diagnostic techniques in Chapter 2 and the integration of all these techniques in the WHO classification in Chapter 3. The cytochemical stains most often employed in acute leukaemia are summarized in Table 1.1 [7,8].

Patients may be assigned to the same or different subgroups depending on the characteristics studied and the criteria selected for separating subgroups. All classifications necessarily have an element of arbitrariness, particularly since they need to incorporate cut-off points for continuous variables such as the percentage of cells falling into a defined morphological category, positivity for a certain cytochemical reaction, or the presence of a certain immunological marker. An ideal classification of acute leukaemia must be biologically relevant. If it is to be useful to the clinical haematologist, as well as to the research scientist, it should also be readily reproducible and easily and widely applicable. Rapid categorization should be possible so that therapeutic decisions can be based on the classification. The classification should be widely acceptable and should change as little as possible over time so that valid comparisons can be made between different groups of patients. Ideal classifications of acute leukaemia do not yet exist, although many have been proposed.

The nature and classification of the myelodysplastic syndromes

The myelodysplastic syndromes are a group of myeloid neoplasms that are closely related to AML and in some cases precede it. Like AML, they result from mutation of a multipotent or, occasionally, a pluripotent haemopoietic stem cell. They differ in that haemopoiesis is ineffective, i.e. there is a normocellular or hypercellular bone marrow but despite this there is peripheral cytopenia as a result of an acquired intrinsic defect in myeloid maturation; there is an increased rate of death of precursor cells in the bone marrow (by a process known as programmed cell death or apoptosis) leading to

Table 1.1 Cytochemical stains of use in the diagnosis and classification of acute leukaemia [7,8].

Cytochemical stain	Specificity
Myeloperoxidase (MPO)	Stains primary and secondary granules of cells of neutrophil lineage, eosinophil granules (granules appear solid), granules of monocytes and Auer rods; granules of normal mature basophils do not stain
Sudan black B (SBB)	Stains primary and secondary granules of cells of neutrophil lineage, eosinophil granules (periphery of granule may stain or granules may appear to have a solid core), granules of monocytes and Auer rods; basophil granules are usually negative but sometimes show metachromatic staining (red/purple)
Naphthol AS-D chloroacetate esterase (chloroacetate esterase (CAE), 'specific' esterase)	Stains neutrophil and mast cell granules; Auer rods are usually negative except in acute myeloid leukaemia associated with t(15;17) and t(8;21)
α-naphthyl acetate esterase (ANAE) ('non-specific' esterase)	Stains monocytes and macrophages, megakaryocytes and platelets, most T lymphocytes and some T lymphoblasts (focal)
α-naphthyl butyrate esterase (ANBE) ('non-specific' esterase)	Stains monocytes and macrophages; variable staining of T lymphocytes
Periodic acid–Schiff (PAS)*	Stains cells of neutrophil lineage (granular, increasing with maturation), leukaemic promyelocytes (diffuse cytoplasmic), eosinophil cytoplasm (but not granules), basophil cytoplasm (blocks), monocytes (diffuse plus granules), megakaryocytes and platelets (diffuse plus granules), some T and B lymphocytes, and many leukaemic blast cells (blocks, B more than T)
Acid phosphatase*	Stains neutrophils, most T lymphocytes, T lymphoblasts (focal); variable staining of eosinophils, monocytes and platelets; strong staining of macrophages, plasma cells and megakaryocytes and some leukaemic megakaryoblasts
Toluidine blue	Stains basophil and mast cell granules
Perls' stain	Stains haemosiderin in erythroblasts, macrophages and, occasionally, plasma cells

* These cytochemical stains are largely redundant if immunophenotyping is available.

a failure of production of adequate numbers of normal mature cells. MDS is also characterized by morphologically abnormal maturation, referred to as dysplasia. However, it should be noted that dysplasia is not specific for MDS, nor even for a myeloid neoplasm. MDS evolves into AML as a result of further mutations that interfere with myeloid maturation leading to a progressive accumulation of blast cells. Not only may MDS evolve into AML, but patients presenting with apparently *de novo* AML may have associated dysplastic features. AML evolving from MDS and AML with associated dysplasia are likely to be closely related conditions. MDS is very heterogeneous, in some patients persisting unchanged for many years and in others leading to death from acute leukaemia or from the complications of bone marrow failure in a relatively short period of time. An adequate classification of MDS must therefore be directed at recognizing categories of disease that differ in prognosis or that indicate a particular, sometimes relatively specific, choice of treatment. The diagnosis and classification of this group of disorders is dealt with in detail in Chapter 4.

The nature and classification of chronic myeloid leukaemias

The chronic myeloid leukaemias can result from a mutation either in a multipotent myeloid stem cell or in a pluripotent lymphoid–myeloid stem cell.

In contrast to the majority of cases of AML, they are characterized by an increased peripheral blood count of mature granulocytes. Usually neutrophils predominate but often there is also an increase in eosinophils and basophils; less often the dominant cell is the eosinophil. Monocytes may also be increased. When the leukaemic clone derives from a pluripotent stem cell, the lymphoid component may be apparent before the myeloid component, simultaneously or subsequently. Irrespective of the timing of the appearance of the lymphoid component, the lymphoid cells are immature and their appearance represents evolution of the disease, known as acute transformation.

The chronic myeloid leukaemias are classified partly on morphological criteria, which in the past were supplemented by cytochemistry (a neutrophil alkaline phosphatase score). However, when a specific cytogenetic or molecular genetic abnormality has been found to characterize a subtype of chronic myeloid leukaemia it becomes of considerable importance to incorporate this into any scheme of classification. A crucial distinction is between chronic myeloid leukaemias with and without a translocation between chromosomes 9 and 22 that leads to the formation of an abbreviated chromosome 22 known as the Philadelphia (Ph) chromosome. Chronic myeloid leukaemia with t(9;22)(q34;q11.2) is variously referred to as 'chronic granulocytic leukaemia', 'chronic myelogenous leukaemia', 'chronic myelogenous leukaemia, *BCR-ABL1* positive' and 'chronic myeloid leukaemia'.

The chronic myeloid leukaemias are similar in nature to myeloproliferative neoplasms (MPN) such as polycythaemia vera, essential thrombocythaemia and the conceptually more complex 'idiopathic' or 'primary' myelofibrosis. In these conditions differentiation is not predominantly to granulocytic cells but to erythrocytes in polycythaemia vera, to platelets in essential thrombocythaemia, and to all myeloid lineages in primary myelofibrosis. The distinguishing features of myelofibrosis are extramedullary haemopoiesis and myelofibrosis, which is not actually either 'primary' or 'idiopathic' but is reactive to the myeloid neoplasm. These other MPN can undergo clonal evolution, one result of which can be Ph-negative chronic myeloid leukaemia.

The chronic myeloid leukaemias are discussed in detail in Chapter 5.

The nature and classification of the myelodysplastic/myeloproliferative neoplasms

The chronic myeloid leukaemias and other MPN are characterized by effective proliferation of myeloid cells and increased numbers of end cells, whereas the MDS are characterized by ineffective proliferation, morphological dysplasia and inadequate numbers of end cells of one or more lineages. However, conditions exist in which there is effective proliferation of cells of one lineage and ineffective proliferation of cells of another lineage. If a condition meets the criteria for both MDS and MPN it is classified as a myelodysplastic/myeloproliferative neoplasm (MDS/MPN). If these overlap syndromes also have a high white blood cell count (WBC) they can legitimately be regarded as a form of (Ph-negative) chronic myeloid leukaemia. Juvenile myelomonocytic leukaemia (JMML), atypical chronic myeloid leukaemia (aCML) and chronic myelomonocytic leukaemia (CMML) are subtypes of MDS/MPN.

The nature and classification of lymphoid neoplasms

Lymphoid neoplasms can be categorized in two ways, according to the immaturity of the cell or according to the presence of absence of 'leukaemia' as a key feature of a type of disease. A lymphoid leukaemia is a neoplasm in which the predominant manifestations are in the blood and bone marrow, whereas the term 'lymphoma' refers to a disease characterized by a neoplastic proliferation of cells of lymphoid origin in organs and tissues such as lymph nodes, spleen, thymus and skin.

Lymphoid neoplasms may be characterized by lymphoblasts, cells that are cytologically and immunophenotypically immature. If lymphoblasts are present in the bone marrow, with or without overspill into the blood, the condition is usually referred to as 'acute lymphoblastic leukaemia' (ALL). Primary infiltration of other lymphoid organs or tissues by lymphoblasts is referred to as 'lymphoblastic lymphoma'. In either case the lymphoblasts can be of either B lineage or T lineage, although ALL is more often of B lineage and lymphoblastic lymphoma more often of T. In the 2008

WHO classification, lymphoid precursor neoplasms are designated 'B lymphoblastic leukaemia/lymphoma' and 'T lymphoblastic leukaemia/lymphoma'.

With regard to lymphoid neoplasms in which the neoplastic cells are immunophenotypically mature, a given condition is regarded as 'leukaemia' or 'lymphoma' according to the usual manifestations of the disease. However, again there is overlap. Thus the most common leukaemia of mature lymphoid cells, chronic lymphocytic leukaemia, has a tissue counterpart designated 'small lymphocytic lymphoma' in which the peripheral blood lymphocyte count is not elevated. A rare subtype of mature T-cell neoplasm is designated adult T-cell leukaemia/lymphoma because about 90% of patients present with leukaemia and about 10% with lymphoma without circulating neoplastic cells. Conditions that are predominantly lymphomas can also have a leukaemic phase when there is extensive disease. This is often the case with mantle cell lymphoma and sometimes with follicular lymphoma. It should be noted that leukaemia and lymphomas of immunophenotypically mature lymphocytes do not necessarily have cells that resemble normal mature lymphocytes cytologically. The neoplastic cells may be very large and appear very abnormal. They are also not necessarily clinically indolent; some, such as Burkitt lymphoma, are as clinically aggressive as acute leukaemia.

The FAB classifications

Defining a blast cell

The enumeration of blasts in the bone marrow is crucial in the diagnosis of acute leukaemia and the definition of a blast cell is therefore important. Whether immature myeloid cells containing small numbers of granules are classified as blasts is a matter of convention. The FAB group chose to classify such cells as myeloblasts rather than promyelocytes. They recognized two types of myeloblast [9]. Type I blasts lack granules and have a diffuse chromatin pattern, a high nucleocytoplasmic ratio and usually prominent nucleoli. Type II blasts resemble type I blasts except for the presence of a few azurophilic granules and a somewhat lower nucleocytoplasmic ratio. Type II blast cells may contain Auer rods (see page 16) rather than granules; less often they contain large rectangular crystals [10] or large inclusions (pseudo-Chédiak–Higashi inclusions). Auer rods and pseudo-Chédiak–Higashi granules may coexist in the same blast cell. Other groups have proposed accepting a type III blast, which has more than 'scanty' granules but otherwise has no features of a promyelocyte [11].

More recently the International Working Group on Morphology of MDS (IWGM-MDS) has revised the definition of a blast cell, accepting as blasts, cells that have more than scanty granules but that do not have other characteristics of promyelocytes [12]. They have divided blast cells into 'agranular blasts' and 'granular blasts'.

Cells are categorized as promyelocytes rather than type II/III or granular blasts when they develop an eccentric nucleus, more abundant cytoplasm, a Golgi zone and some chromatin condensation (but with the retention of a nucleolus). The cytoplasm, except in the pale Golgi zone, remains basophilic. Cells that have few or no granules, but that show the other characteristics of promyelocytes, are regarded as hypogranular or agranular promyelocytes rather than as blasts. Examples of cells classified as types I, II and III blasts and as promyelocytes are shown in Figs 1.1–1.4. The great majority of lymphoblasts lack granules and are therefore type I blasts; they resemble myeloblasts but are often smaller with scanty cytoplasm and may show some chromatin condensation (see Table 1.11). Type II and III (granular) blast cells are generally myeloid (although some lymphoblasts have a few (myeloperoxidase-negative) granules and are thus type II blasts).

Monoblasts (Fig. 1.4a) differ from myeloblasts in being larger with more voluminous cytoplasm. The cytoplasm is moderately to markedly basophilic and may have fine granules or vacuoles. The nucleus is round or somewhat oval with a dispersed chromatin pattern and often a large single nucleolus. The cell may be round or have an irregular cytoplasmic margin.

Defining a promonocyte

The promonocyte has been described in similar terms by the FAB group and in the WHO classification. Since the WHO classification regards the promonocyte as a 'blast equivalent' in the

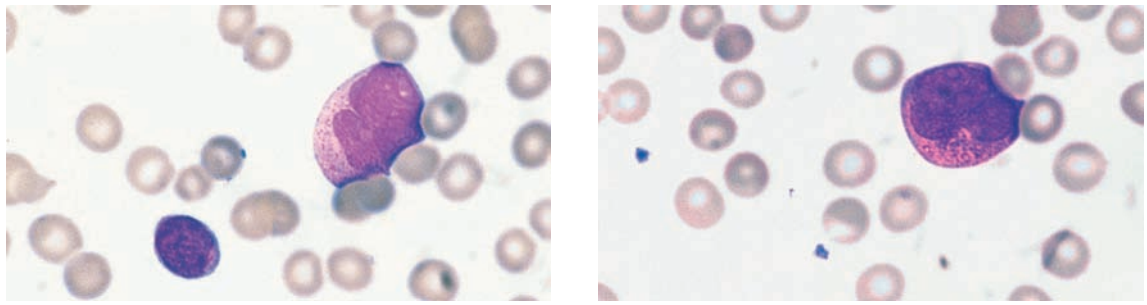


Fig. 1.1 The peripheral blood (PB) film of a patient with acute myeloid leukaemia (AML) showing: (a) a type II blast with scanty azurophilic granules; (b) a promyelocyte with more numerous granules and a Golgi zone in the indentation of the nucleus. May–Grünwald–Giemsa (MGG) ×100.

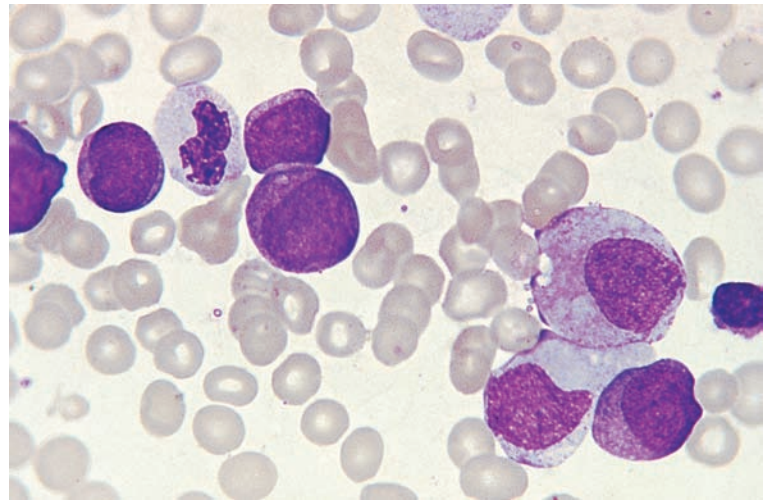


Fig. 1.2 Bone marrow (BM) film of a patient with AML (FAB (French–American–British classification) M2/t(8;21)) showing a cell that has scanty granules but nevertheless would be classified as a promyelocyte rather than a blast because of its low nucleocytoplasmic ratio; defective granulation of a myelocyte and a neutrophil is apparent. Type I and type II blasts are also present. MGG ×100.

diagnosis of myeloid neoplasms, its recognition has become of considerable importance. The misclassification of immature or abnormal monocytes as promonocytes can lead to a disease being categorized as AML rather than as MDS or CMML.

A promonocyte (Figs 1.4b and 1.5) is a large cell with an irregular or convoluted nucleus. The cytoplasm is weakly or moderately basophilic. The cytoplasm may be vacuolated or contain granules. The chromatin pattern is diffuse, like that of a monoblast. A nucleolus with similar characteristics may be present or the nucleolus may be smaller. It is the features of the nucleus that permit a distinction between a monoblast and a promonocyte; both have the same delicate or dispersed chromatin pattern but the monoblast has a regular nucleus whereas that of the promonocyte is irregular.

Promonocytes must be distinguished from immature or atypical monocytes, which have some chromatin condensation and rarely have nucleoli, these being the essential features that differentiate them from promonocytes. They have lobulated or indented nuclei and cytoplasm that shows variable basophilia and may have granules or vacuoles; the cytoplasmic outline may be irregular.

The FAB classification of acute leukaemia

The FAB classification of acute leukaemia was first published in 1976 and was subsequently expanded, modified and clarified [1–5]. It deals with both diagnosis and classification.

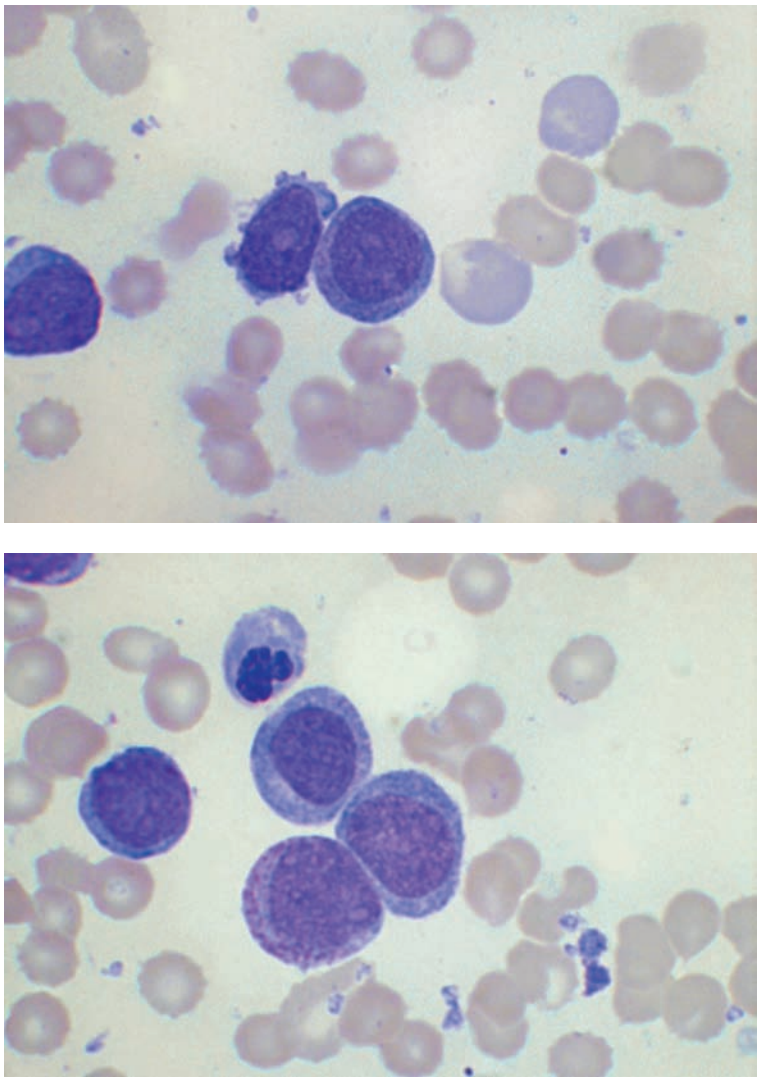
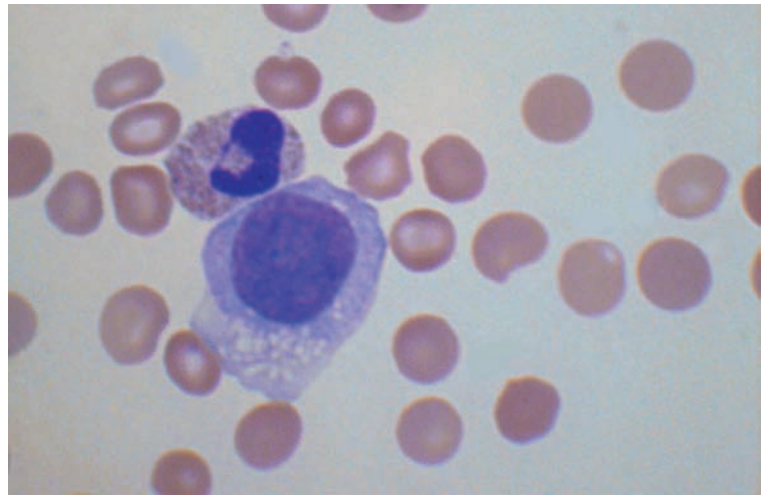


Fig. 1.3 BM film from a patient with FAB type M2 AML showing: (a) a type I blast cell (left of centre) and a type II blast cell with scanty granules (centre); (b) a type II (granular) blast cell with numerous granules but with a central nucleus and no Golgi zone; there are also three type I blast cells and a dysplastic erythroblast. MGG ×100.

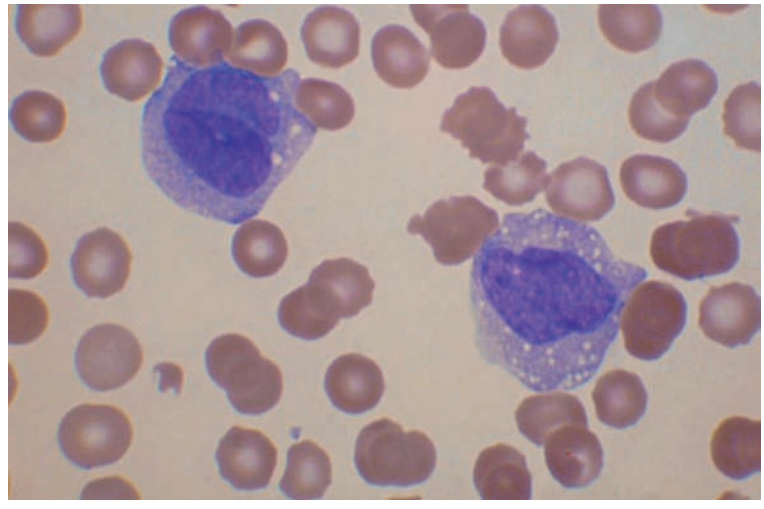
Diagnosing acute leukaemia

The diagnosis of acute leukaemia usually starts from a clinical suspicion. It is uncommon for this diagnosis to be incidental, resulting from the performance of a blood count for a quite different reason. Clinical features leading to suspicion of acute leukaemia include pallor, fever or other signs of infection, pharyngitis, petechiae and other haemorrhagic manifestations, bone pain, hepatomegaly, splenomegaly, lymphadenopathy, gum hypertrophy and skin infiltration. A suspicion of acute leukaemia generally leads to a blood count and film being performed and, if this shows a relevant abnormality, to a bone marrow aspiration. The diagnosis then rests on an assessment of the peripheral blood and bone marrow. Radiological features can also be of value, with a mediastinal mass being strongly suggestive of T-lineage ALL.

The peripheral blood in AML usually shows leucocytosis, anaemia and thrombocytopenia. The leucocytosis reflects the presence of circulating blast cells, while the number of neutrophils is usually reduced and few cells of intermediate stages of maturation are seen (*hiatus leukaemicus*). In some



(a)



(b)

Fig. 1.4 BM film from a patient with FAB type M5 AML showing: (a) a monoblast and a neutrophil; (b) two promonocytes. MGG ×100.

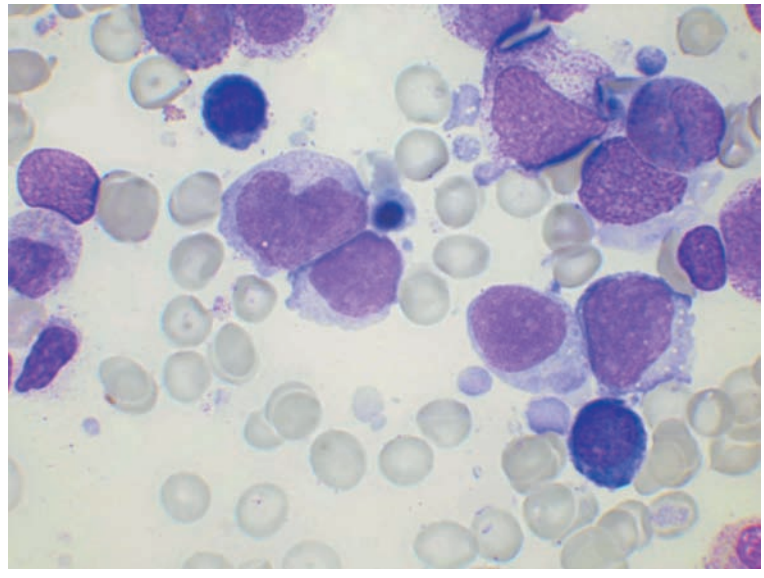


Fig. 1.5 BM film from a patient with FAB type M5 AML showing a promonocyte and three monoblasts; the promonocyte has an irregular nucleus but otherwise is very similar to the three monoblasts. MGG ×100.

patients the total WBC is normal or low and, in the latter group, circulating blast cells may be infrequent or even absent. In a minority of patients, there are increased eosinophils and, considerably less often, increased basophils. There may be evidence of dysplastic maturation such as poikilocytosis and macrocytosis, hypolobated or agranular neutrophils or hypogranular/agranular or giant platelets.

The peripheral blood film in ALL may show leucocytosis resulting from the presence of considerable numbers of circulating blast cells, but many patients have a normal total leucocyte count and blast cells may be infrequent or even absent. There is usually anaemia, neutropenia or thrombocytopenia but sometimes the neutrophil count, platelet count or even both may be normal and occasionally the platelet count is actually increased. In contrast to AML, the myeloid cells do not show any dysplastic features. A minority of patients have a reactive eosinophilia.

The FAB classification requires that peripheral blood and bone marrow films be examined and that differential counts be performed on both. In the case of the bone marrow, a 500-cell differential count is required. Acute leukaemia is diagnosed if one of the following three features is present:

1 At least 30%* of the total nucleated cells in the bone marrow are blast cells; *or*

2 The bone marrow shows erythroid predominance (erythroblasts ≥50% of total nucleated cells) and at least 30% of non-erythroid cells are blast cells (lymphocytes, plasma cells and macrophages also being excluded from the differential count of non-erythroid cells); *or*

3 The characteristic morphological features of acute promyelocytic leukaemia (see page 21) are present (Fig. 1.6).

Cases of ALL will be diagnosed on the first criterion since erythroid hyperplasia does not occur in this condition, but the diagnosis of all cases of AML requires application also of the second and third criteria. The bone marrow in acute leukaemia is usually hypercellular, or at least normocellular, but this is not necessarily so since some cases meet the above criteria when the bone marrow is hypocellular.

* It should be noted that the criterion of at least 30% blast cells has been altered, in the WHO classification, to at least 20% blast cells (see page 115).

Distinguishing between acute myeloid and acute lymphoblastic leukaemias

The diagnosis of acute leukaemia using FAB criteria requires that bone marrow blast cells (type I plus type II) constitute at least 30% either of total nucleated cells or of non-erythroid cells. The further classification of acute leukaemia as AML or ALL is of critical importance. When the FAB classification was first proposed, tests to confirm the nature of lymphoblasts were not widely available. The group therefore defined as AML cases in which at least 3% of the blasts gave positive reactions for myeloperoxidase (MPO) or with Sudan black B (SBB). Cases that appeared to be non-myeloid were classed as 'lymphoblastic'. The existence of cases of AML in which fewer than 3% of blasts gave cytochemical reactions appropriate for myeloblasts or monoblasts was not established at this stage, and no such category was provided in the initial FAB classification. In the 1980s and 1990s the wider availability and application of immunological markers for B- and T-lineage lymphoblasts, supplemented by ultrastructural cytochemistry and the application of molecular biological techniques to demonstrate rearrangements of immunoglobulin and T-cell receptor genes, demonstrated that the majority of cases previously classified as 'lymphoblastic' were genuinely lymphoblastic but that a minority were myeloblastic with the blast cells showing only minimal evidence of myeloid differentiation.† These latter cases were designated M0 AML [5]. It should be noted that SBB is more sensitive than MPO in the detection of myeloid differentiation and more cases will be categorized as M1 rather than M0 if it is used [13].

Correct assignment of patients to the categories of AML and ALL is very important for prognosis and choice of therapy. Appropriate tests to make this distinction must therefore be employed. Despite the advances in immunophenotyping, cytochemical reactions remain useful in the diagnosis of AML

† In discussing the FAB classification I have used the terms 'differentiation' and 'maturation' in the sense in which they were used by the FAB group, that is with differentiation referring to an alteration in gene expression that commits a multipotent stem cell to one pathway or lineage rather than another, and maturation indicating the subsequent changes within this cell and its progeny as they mature towards end cells of the lineage.

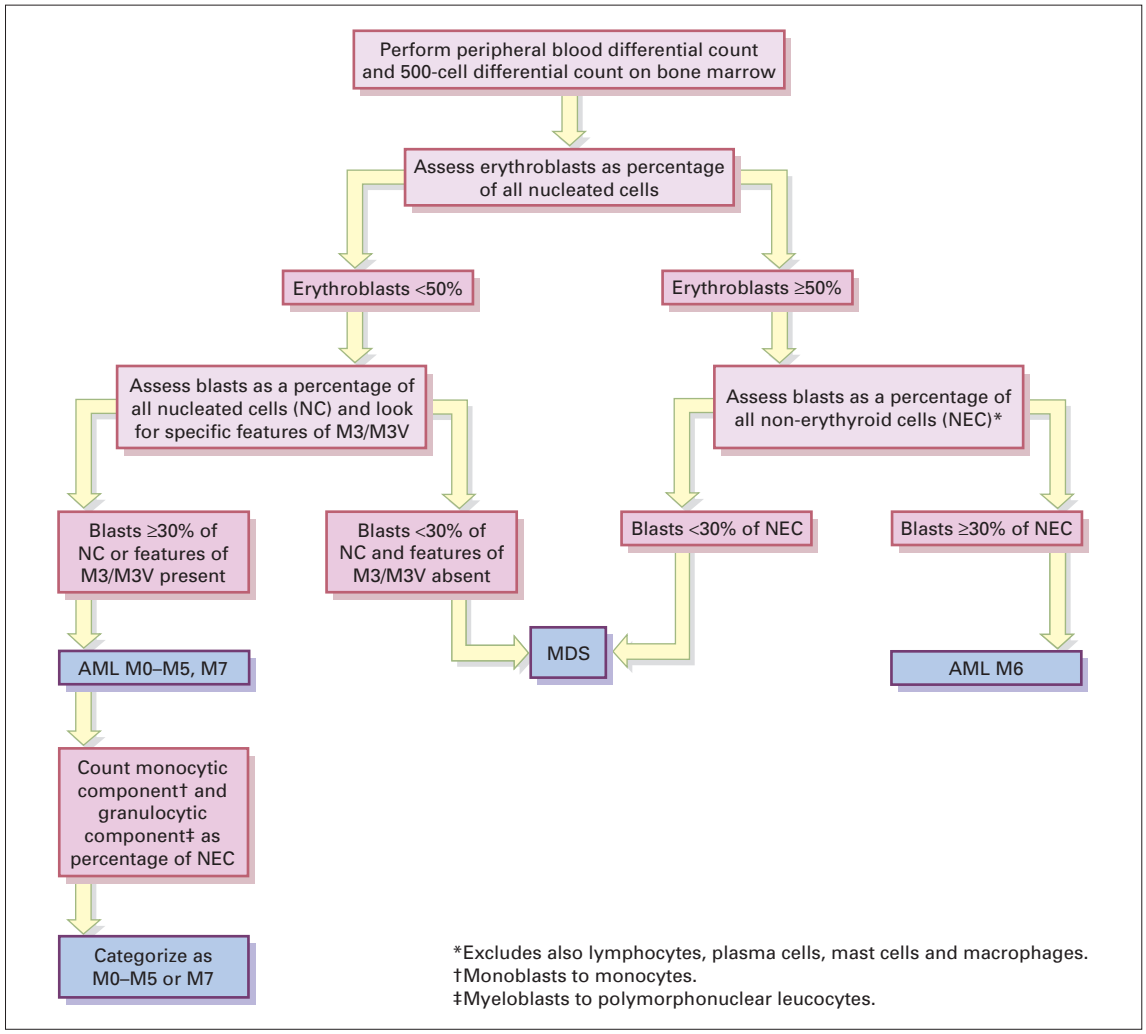


Fig. 1.6 A flow chart illustrating the diagnosis of AML and its distinction from the myelodysplastic syndromes (MDS), according to the FAB diagnostic criteria.

[14]. Cytochemical demonstration of MPO activity can also give prognostic information, since a higher percentage of MPO-positive blasts is strongly associated with a better prognosis [15]. The FAB group recommended the use of MPO, SBB and non-specific esterase (NSE) stains. If cytochemical reactions for myeloid cells are negative, a presumptive diagnosis of ALL should be confirmed by immunophenotyping. When immunophenotyping is available the acid phosphatase reaction and the periodic acid–Schiff (PAS) reaction (the latter identifying a variety of carbohydrates including glycogen) are no longer indicated for the diagnosis of ALL. When cytochemical reactions indicative of myeloid differentiation and immunophenotyping for lymphoid antigens are both negative, immunophenotyping to demonstrate myeloid antigens and thus identify cases of M0 AML is necessary; the panel of antibodies used for characterizing suspected acute leukaemia normally includes antibodies directed at both lymphoid and myeloid antigens so that the one procedure will identify both M0 AML and ALL. It should be noted that when individuals with an inherited MPO deficiency develop AML, leukaemic

cells will give negative reactions for both MPO and SBB.

Defining remission

Morphological remission in acute leukaemia is often defined as the absence of clinical evidence of leukaemia (e.g. no extramedullary disease) with bone marrow blast cells being less than 5%, no Auer rods being present, the neutrophil count being at least 1×10^9/l and the platelet count being at least 100×10^9/l [16]. A bone marrow blast percentage of less than 5% has been validated [17]. Sometimes the definition includes a provision that these criteria are met for a minimum of 1 month or that, if immunophenotypic analysis is carried out, there is no persistence of a leukaemia-associated immunophenotype. A more strictly defined remission is a cytogenetic remission, which requires there to be no cytogenetic evidence of a persisting leukaemic clone [16]. Similarly, a molecular complete remission requires that there be no molecular evidence of minimal residual disease [16].

The incidence of acute leukaemia

Acute myeloid leukaemia has a low incidence in childhood, less than one case per 100 000/year. Among adults the incidence rises increasingly rapidly with age, from approximately 1/100 000/year in the fourth decade to approximately 10/100 000/year in those over 70 years. AML is commoner in males than in females. ALL is most common in childhood, although cases occur at all ages. In children up to the age of 15 years the overall incidence is of the order of 2.5–3.5 /100 000/year; the disease is more common in males than in females. In childhood, ALL is more common than AML, except under the age of 1 year. ALL has also been observed to be more common in Caucasians than in those of African ancestry, but this appears to be related to environmental factors rather than being a genetic difference since the difference disappears with an alteration in socioeconomic circumstances.

The FAB categories and other morphological categories of acute myeloid leukaemia

Once criteria for the diagnosis of AML have been met and cases have been correctly assigned to the broad categories of myeloid or lymphoid, further classification can be carried out. The FAB group suggested that this be based on a peripheral blood differential count and a 500-cell bone marrow differential count, supplemented when necessary by cytochemistry, studies of lysozyme concentration in serum or urine, and immunophenotyping; with the greater availability of immunophenotyping, measurement of lysozyme concentration is no longer in current use. Broadly speaking, AML is categorized as acute myeloblastic leukaemia without (M1) and with (M2) maturation, acute hypergranular promyelocytic leukaemia and its variant (M3 and M3V), acute myelomonocytic leukaemia (M4), acute monoblastic (M5a) and monocytic (M5b) leukaemia, acute erythroleukaemia (M6) and acute megakaryoblastic leukaemia (M7). M0 is AML without maturation and with minimal evidence of myeloid differentiation. In addition to the above categories there are several very rare types of AML that are not included in the FAB classification. These include mast cell leukaemia and Langerhans cell leukaemia. In addition, the diagnosis of hypoplastic AML requires consideration. Transient abnormal myelopoiesis of Down syndrome (see page 164) should also be regarded as a variant of AML.

Acute myeloid leukaemia with minimal evidence of myeloid differentiation: M0 acute myeloid leukaemia

The FAB criteria for the diagnosis of M0 AML are shown in Table 1.2 and the morphological and immunocytochemical features are illustrated in Figs 1.7 and 1.8. The blasts in M0 AML usually resemble M1 myeloblasts or L2 lymphoblasts (see

Table 1.2 Criteria for the diagnosis of acute myeloid leukaemia of M0 category (acute myeloid leukaemia with minimal evidence of myeloid differentiation).

- Blasts ≥30% of bone marrow nucleated cells
- Blasts ≥30% of bone marrow non-erythroid cells*
- <3% of blasts positive for Sudan black B or for myeloperoxidase by light microscopy
- Blasts demonstrated to be myeloblasts by immunological markers or by ultrastructural cytochemistry

* Exclude also lymphocytes, plasma cells, macrophages and mast cells from the count.

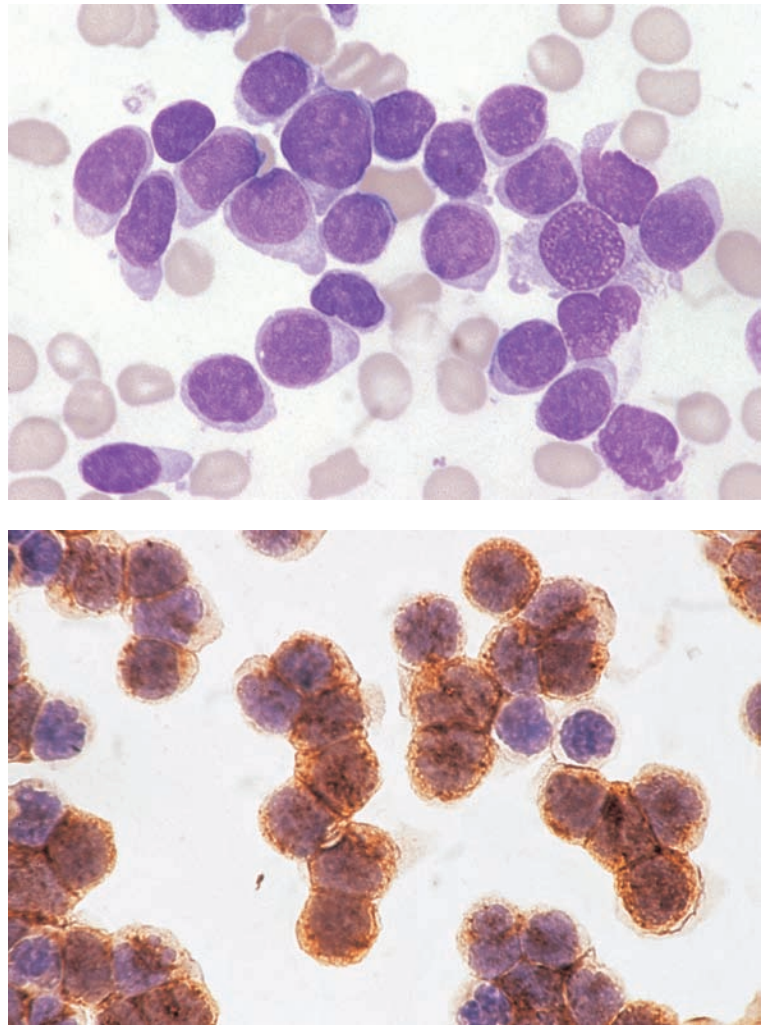


Fig. 1.7 PB and BM preparations from a patient with FAB M0 AML. (a) BM film stained by MGG showing agranular blasts. MGG ×100. (b) Immunoperoxidase reaction of PB cells in a cytospin preparation stained with a CD13 monoclonal antibody (McAb) showing many strongly positive blasts; the blasts were also positive for CD34, HLA-DR and terminal deoxynucleotidyl transferase (TdT). Immunoperoxidase ×100.

page 49) but in a minority of cases they resemble the monoblasts of M5 AML. Associated dysplastic features in erythroid and megakaryocyte lineages may provide indirect evidence that a leukaemia is myeloid not lymphoid. Dysplastic features are present in up to a quarter of cases. Definite evidence of myeloid differentiation that permits assignment to this category may be provided by the following:

1 The demonstration of ultrastructural features of cells of granulocytic lineage, e.g. characteristic basophil granules [18–23] (Table 1.3).

2 The demonstration of MPO activity by ultrastructural cytochemistry [19,24,25] (Table 1.4; Fig. 1.9).

3 The demonstration of MPO protein by immunocytochemistry with an anti-MPO monoclonal antibody.

4 The demonstration of other antigens characteristic of myeloid cells by the use of monoclonal antibodies such as CD13, CD14, CD15, CD33, CD64, CD65 and CD117 (but without expression of platelet-specific antigens, which would lead to the case being categorized as AML M7).

Although not included in the criteria suggested by the FAB group, the demonstration of messenger RNA (mRNA) for MPO has also been suggested as a criterion for recognition of myeloid differentiation

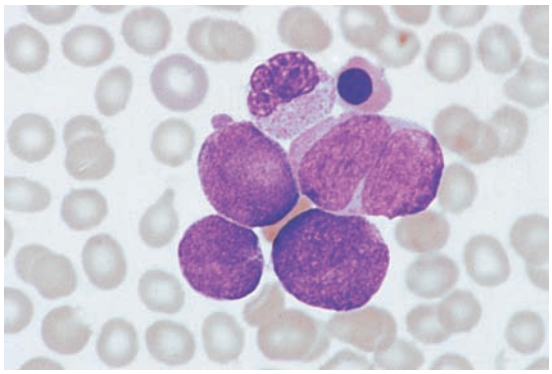


Fig. 1.8 BM film of a patient with FAB M0 AML showing agranular pleomorphic blasts with a high nucleocytoplasmic ratio; the presence of a neutrophil with a nucleus of abnormal shape suggests the correct diagnosis. MGG ×100.

[26] but its expression may not be restricted to myeloid cells [27].

Immunophenotyping is now widely used for identifying cases of M0 AML and as a consequence ultrastructural examination and ultrastructural cytochemistry are rarely used. However, these techniques remain useful for the identification of immature cells of basophil, mast cell and eosinophil lineage. Immunophenotyping shows that the most specific lymphoid markers – CD3 and CD22 – are not expressed in M0 AML but there may be expression of less specific lymphoid-associated antigens such as CD2, CD4, CD7, CD10 and CD19, in addition to CD34, human leucocyte antigen DR (HLA-DR) and terminal deoxynucleotidyl transferase (TdT). CD7 is more often expressed than in other FAB categories of AML [28].

Table 1.3 Ultrastructural characteristics distinguishing blast cells and other immature leukaemic cells from each other [18,19].

Myeloblasts of neutrophil lineage
Small, medium or large granules; sometimes Auer rods, which may be homogeneous or be composed of longitudinal tubules or dense material with a periodic substructure [20]

Promyelocytes of promyelocytic leukaemia
In hypergranular promyelocytic leukaemia the cytoplasm is packed with granules ranging from 120 to 1000 nm in diameter [21,22]; in the variant form of hypergranular promyelocytic leukaemia the granules are much smaller, ranging from 100 to 400 nm, with some cells being packed with granules and other being agranular. Auer rods in promyelocytic leukaemia differ from those in M1 and M2 AML; they are composed of hexagonal structures and have a different periodicity from other Auer rods [22]; microfibrils and stellate configurations of rough endoplasmic reticulum are also characteristic of M3 AML, particularly M3 variant [23]

Myeloblasts of eosinophil lineage
Granules tend to be larger than those of neutrophil series; homogeneous in early cells, in later cells having a crystalline core set in a matrix; sometimes there is asynchrony with granules lacking a central core, despite a mature nucleus. Auer rods similar to those of the neutrophil lineage may be present [20]

*Myeloblasts of basophil or mast cell lineage**
Basophil granules may be any of three types: (i) large, electron-dense granules composed of coarse particles; (ii) pale granules composed of fine particles; or (iii) θ granules, which are small granules containing pale flocculent material and bisected by a membrane [19]. Mast cell precursors sometimes have granules showing the scrolled or whorled pattern that is characteristic of normal mast cells

Monoblasts and promonocytes
Monoblasts are larger than myeloblasts and cytoplasm may be vacuolated. Granules are smaller and less numerous

Megakaryoblasts
More mature megakaryoblasts show α granules, bull's eye granules and platelet demarcation membranes

Early erythroid precursors
Immature cells can be identified as erythroid when they contain aggregates of ferritin molecules or iron-laden mitochondria or when there is rhopheocytosis (invagination of the surface membrane in association with extracellular ferritin molecules)

* Sometimes in myeloid leukaemias and myeloproliferative neoplasms there are cells containing a mixture of granules of basophil and mast cell type.

Table 1.4 Ultrastructural cytochemistry in the identification of blast cells and other immature cells of different myeloid lineages.

Myeloblasts of neutrophil lineage MPO activity in endoplasmic reticulum, perinuclear space, Golgi zone, granules and Auer rods (if present); detected by standard technique for MPO and by PPO techniques (reviewed in reference 19)
Myeloblasts of eosinophil lineage MPO-positive granules and Auer rods (if present) detected by MPO and PPO techniques
Myeloblasts of basophil or mast cell lineage Granules may be peroxidase positive or negative; endoplasmic reticulum, perinuclear space and Golgi zone are rarely positive; more cases are positive by PPO technique than MPO technique
Promyelocytes of acute promyelocytic leukaemia MPO positivity is seen in granules, Auer rods, perinuclear space and some rough endoplasmic reticulum profiles [23]; strong lysozyme activity of granules and Auer rods is seen in M3 AML, whereas in M3 variant AML activity varies from weak to moderately strong [23]
Monoblasts and promonocytes The first granule to appear in a monoblast is a small, peripheral acid phosphatase-positive granule [24]. MPO activity appears initially in the perinuclear envelope, Golgi apparatus and endoplasmic reticulum. Subsequently, mainly at the promonocyte stage, there are small MPO-positive granules. A PPO technique is more sensitive in the detection of peroxidase-positive granules than an MPO technique. Non-specific esterase activity can also be demonstrated cytochemically
Megakaryoblasts PPO activity in endoplasmic reticulum and perinuclear space only [19,25]
Proerythroblasts PPO-like activity may be present in the Golgi zone

AML, acute myeloid leukaemia; MPO, myeloperoxidase; PPO, platelet peroxidase.

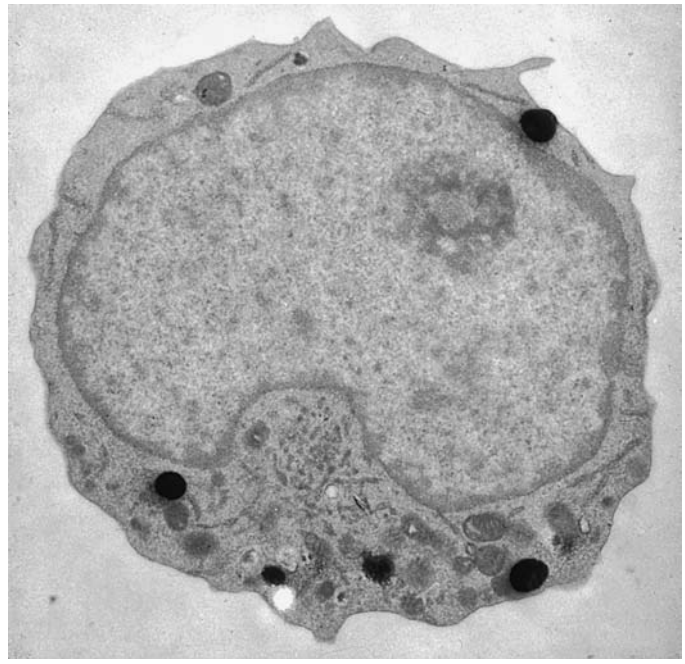


Fig. 1.9 Ultrastructural cytochemistry showing peroxidase-positive granules in a myeloblast. (By courtesy of Professor Daniel Catovsky, London.)

M0 AML has been associated with older age, higher WBC, adverse cytogenetic abnormalities and poor prognosis [28–30]. The molecular genetic abnormalities recognized include a high incidence of mutations of the *RUNX1* (*AML1*) gene, most of which are biallelic [28,31]. In children M0 AML has been associated with a lower WBC, more frequent –5/del(5q), more frequent +21, more frequent hypodiploidy and an inferior outcome [32].

Cytochemical reactions in M0 acute myeloid leukaemia
By definition fewer than 3% of blasts are positive for MPO, SBB and chloroacetate esterase (CAE) since a greater degree of positivity would lead to the case being classified as M1 AML. Similarly, blast cells do not show NSE activity, since positivity would lead to the case being classified as M5 AML. Maturing myeloid cells may show peroxidase deficiency or aberrant positivity for both chloroacetate and non-specific esterases [33].

Acute myeloid leukaemia without maturation: M1 acute myeloid leukaemia
The criteria for diagnosis of M1 AML are shown in Table 1.5 and the cytological features are illustrated in Figs 1.10–1.13. M1 blasts are usually medium to large in size with a variable nucleocytoplasmic ratio, a round or oval nucleus, one or more nucleoli – which range from inconspicuous to prominent – and cytoplasm that sometimes contains Auer rods, a few granules or some vacuoles. Auer rods are crystalline cytoplasmic structures derived from primary granules either just after their formation in the cisternae of the Golgi apparatus or by coalescence of granules within autophagic vacuoles. They were first described by Thomas McCrae in 1905 and a year later by John Auer [34–36]. Auer rods may be seen as cytoplasmic inclusions or, less often, within a cytoplasmic vacuole. Similar structures have been reported in rare myeloid cells in the fetus [37] but otherwise these structures appear to be specific for myeloid neoplasms. In children, the presence of Auer rods has been found to be associated with a better prognosis [38]. In M1 AML the blasts are predominantly type I blasts. In some cases the blasts are indistinguishable from L2 or even L1 lymphoblasts (see page 49).

M1 is arbitrarily separated from M2 AML by the requirement that no more than 10% of non-

Table 1.5 Criteria for the diagnosis of acute myeloid leukaemia of M1 category (acute myeloid leukaemia without maturation).

- Blasts ≥30% of bone marrow cells
- Blasts ≥90% of bone marrow non-erythroid cells*
- ≥3% of blasts positive for peroxidase or Sudan black B
- Bone marrow maturing monocytic component (promonocytes to monocytes) ≤10% of non-erythroid cells
- Bone marrow maturing granulocytic component (promyelocytes to polymorphonuclear leucocytes) ≤10% of non-erythroid cells

* Exclude also lymphocytes, plasma cells, macrophages and mast cells from the count.

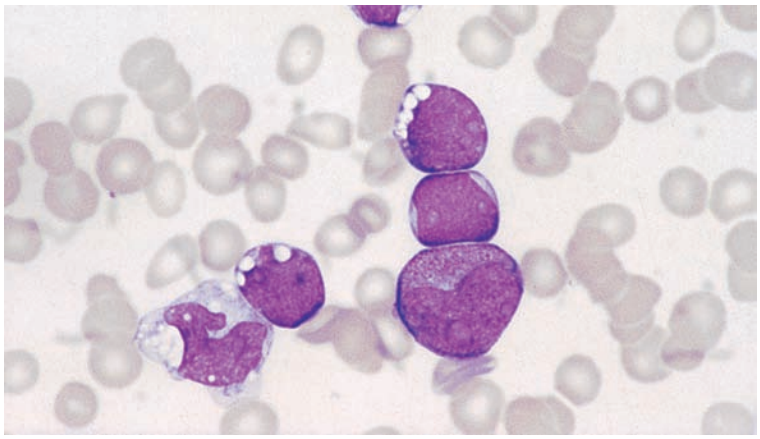


Fig. 1.10 PB film of a patient with FAB M1 AML showing type I and type II blasts, some of which are heavily vacuolated, and a promyelocyte. MGG ×100.

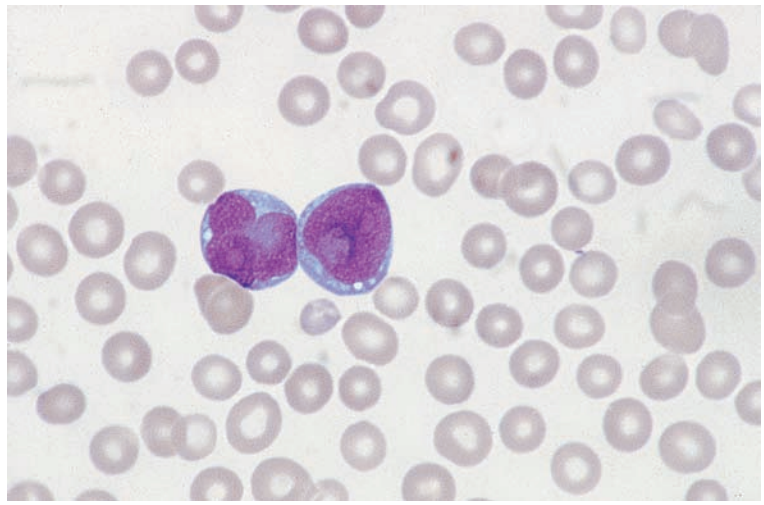


Fig. 1.11 PB film of a patient with FAB M1 AML showing type I blasts with cytoplasmic vacuolation and nuclear lobulation. MGG ×100.

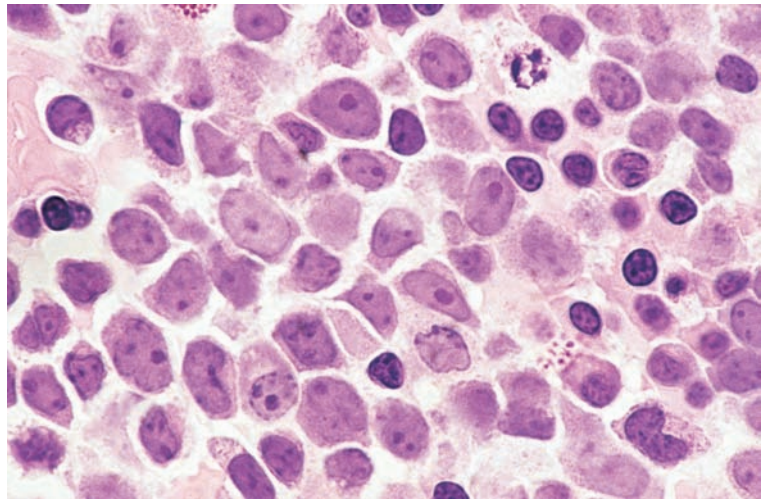


Fig. 1.12 Trephine biopsy section from a patient with FAB M1 AML. The majority of cells present are blasts with a high nucleocytoplasmic ratio and prominent nucleoli; there are also some erythroblasts. Resin embedded, haematoxylin and eosin (H&E) ×100.

erythroid cells in the bone marrow belong to the maturing granulocytic component (promyelocytes to neutrophils).

The M1 category accounts for 15–20% of AML.

Cytochemical reactions in M1 acute myeloid leukaemia
By definition, M1 AML has a minimum of 3% of blasts that are positive for MPO or SBB. Hayhoe and Quaglino [8] found that the SBB reaction is a more sensitive marker of early granulocyte precursors than MPO. M1 blasts are usually positive for CAE, although this marker is usually less sensitive than either MPO or SBB in the detection of neutrophilic differentiation. Myeloblasts give a weak or negative reaction for a number of esterases that are more characteristic of the monocyte lineage, and that are collectively referred to as non-specific esterases. In the case of α-naphthyl acetate esterase (ANAE) and α-naphthyl butyrate esterase (ANBE) the reaction is usually negative, whereas in the cases of naphthol AS-D acetate esterase (NASDA) there is usually a weak fluoride-resistant reaction. Myeloblasts show diffuse acid phosphatase activity, which varies from weak to strong. The PAS reaction is usually negative, but may show a weak diffuse reaction with superimposed fine granular positivity.

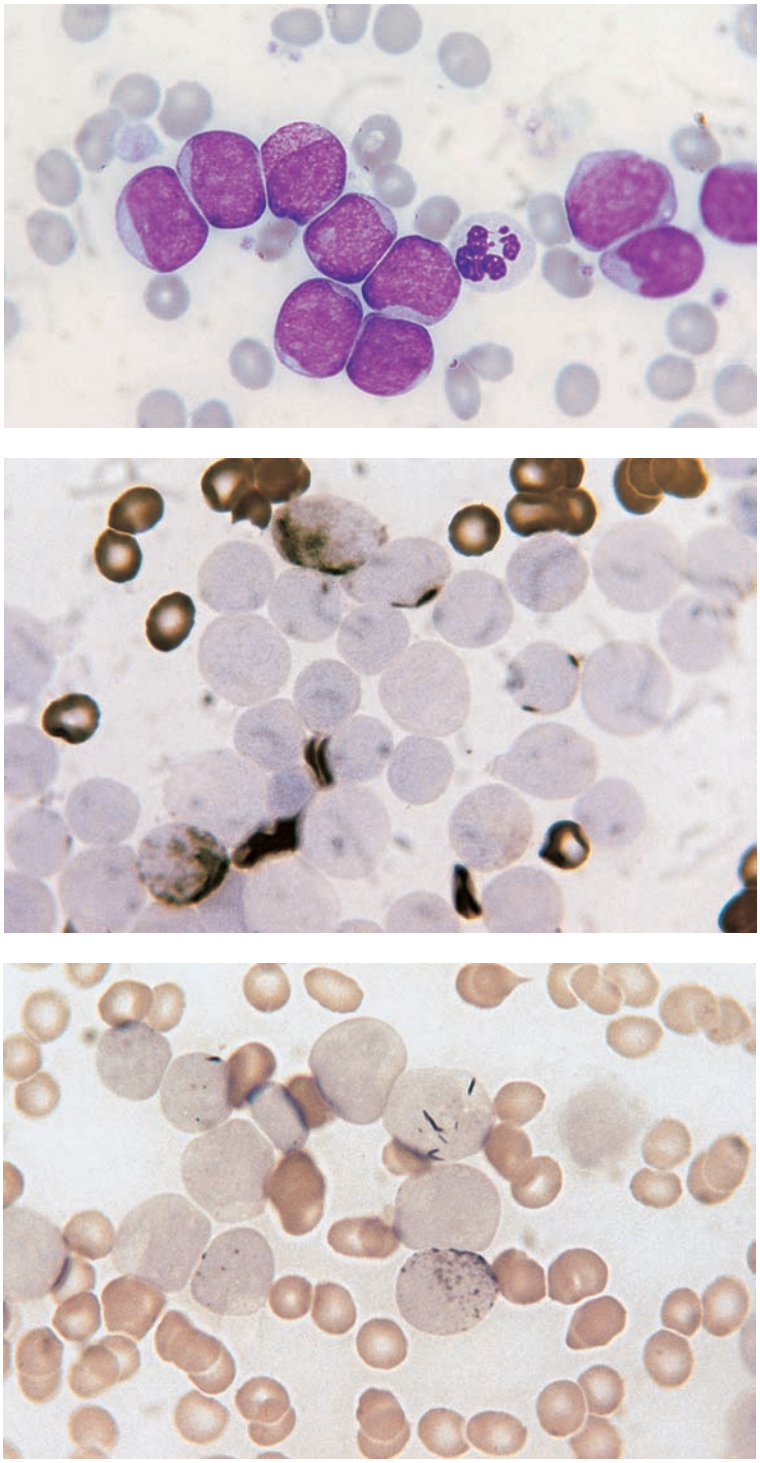
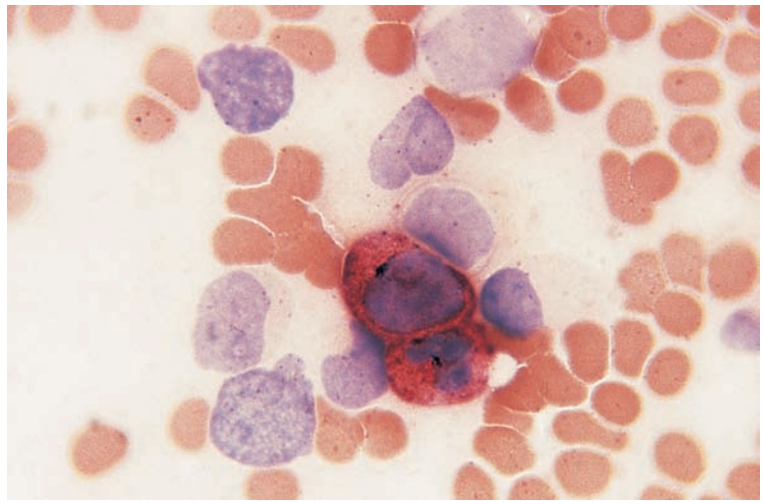


Fig. 1.13 Cytochemical reactions in a patient with M1 AML. (a) MGG-stained PB film showing largely type I blasts which in this patient are morphologically similar to lymphoblasts. One leukaemic cell is heavily granulated and would therefore be classified as a promyelocyte; this cell and the presence of a hypogranular neutrophil suggest that the correct diagnosis is M1 AML. MGG ×100. (b) Myeloperoxidase (MPO)-stained BM film showing two leukaemic cells with peroxidase-positive granules and two with Auer rods. MPO ×100. (c) Sudan black B (SBB) stain of a BM film showing some blasts with Auer rods and some with granules. SBB ×100.



(d)

Fig. 1.13 (*Continued*)
(d) Chloroacetate esterase (CAE) stain of a BM film showing a positive neutrophil and a positive blast; other blasts present are negative. CAE ×100.

Auer rods give positive MPO and SBB reactions and occasionally weak PAS reactions. The reaction for CAE is usually weak or negative [8] except in M2 AML associated with t(8;21) (see page 116) in which Auer rods are often positive for CAE [7]. Although Auer rods are often detectable on a Romanowsky stain, they are more readily detectable on an MPO or SBB stain and larger numbers are apparent. Sometimes they are detectable only with cytochemical stains. Typical cytochemical stains in a case of M1 AML are shown in Fig. 1.13.

Table 1.6 Criteria for the diagnosis of acute myeloid leukaemia of M2 category (acute myeloid leukaemia with maturation).

- Blasts ≥30% of bone marrow cells
- Blasts 30–89% of bone marrow non-erythroid cells
- Bone marrow maturing granulocytic component (promyelocytes to polymorphonuclear leucocytes) >10% of non-erythroid cells
- Bone marrow monocytic component (monoblasts to monocytes) <20% of non-erythroid cells and other criteria for M4 not met

Acute myeloid leukaemia with maturation: M2 acute myeloid leukaemia

The criteria for the diagnosis of M2 AML are shown in Table 1.6. In this context, cells included in the maturing granulocytic category are promyelocytes, myelocytes, metamyelocytes and granulocytes, and also cells that differ cytologically from normal promyelocytes but that are too heavily granulated to be classified as blasts when using FAB criteria. Typical cytological and cytochemical features in M2 AML are shown in Figs 1.14–1.16. In contrast to M1 AML, blasts are often predominantly type II. Auer rods may be present. In children, Auer rods have been associated with a better prognosis [38], probably because of an association between Auer rods and t(8;21) (see page 116). Dysplastic features, such as hypo- or hypergranularity or abnormalities of nuclear shape are common in the differentiating granulocytic component of M2 AML. Maturation of myeloblasts to promyelocytes occurs in both M2 and M3 AML, and promyelocytes are prominent in some cases of M2 AML. Such cases are distinguished from M3 AML by the lack of the specific features of the latter condition (see below). M2 AML is distinguished from M4 AML by the monocytic component in the bone marrow being less than 20% of non-erythroid cells and by the lack of other evidence of significant monocytic differentiation. In most cases of M2 AML, maturation is along the neutrophil pathway but eosinophilic or basophilic maturation occurs in a minority. Such cases may be designated M2Eo or M2Baso. Other morphologically distinctive categories within M2, associated with specific cytogenetic abnormalities, are recognized (see Chapter 3).

The M2 subtype accounts for about 30% of cases of AML.

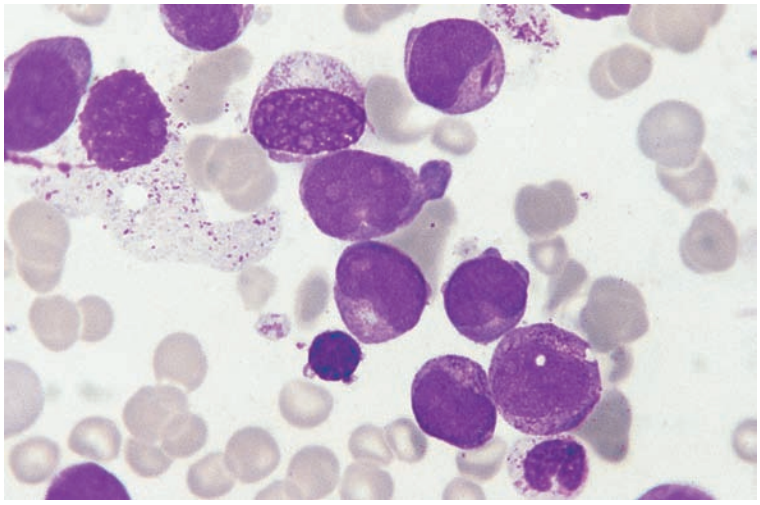
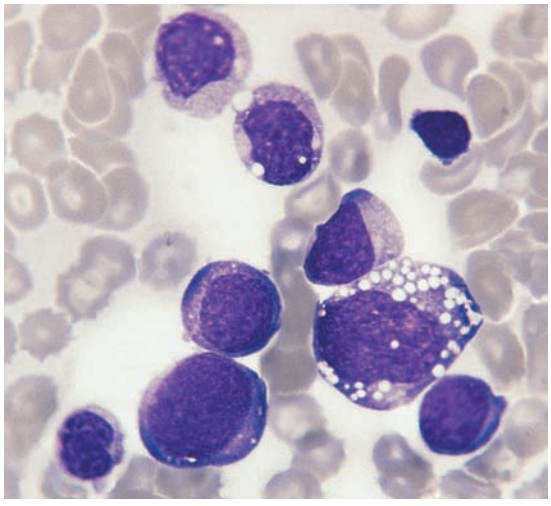
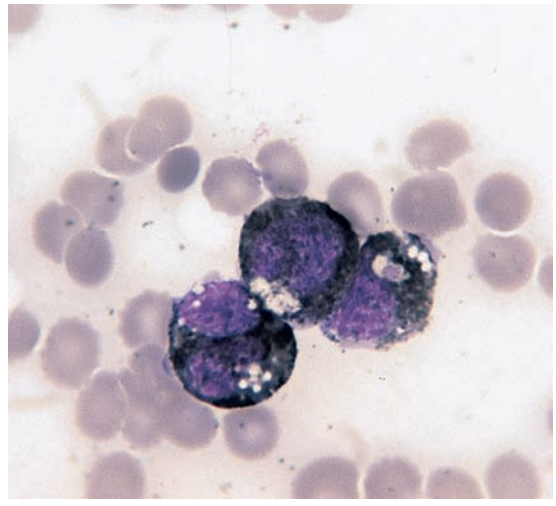


Fig. 1.14 BM film of a patient with FAB M2 AML showing blasts (one of which contains an Auer rod), promyelocytes and a neutrophil. Note the very variable granulation. MGG ×100.

(a)





(b)

Fig. 1.15 BM film of a patient with FAB M2 AML stained by (a) MGG and (b) SBB. In this patient both blasts and maturing cells were heavily vacuolated. ×100.

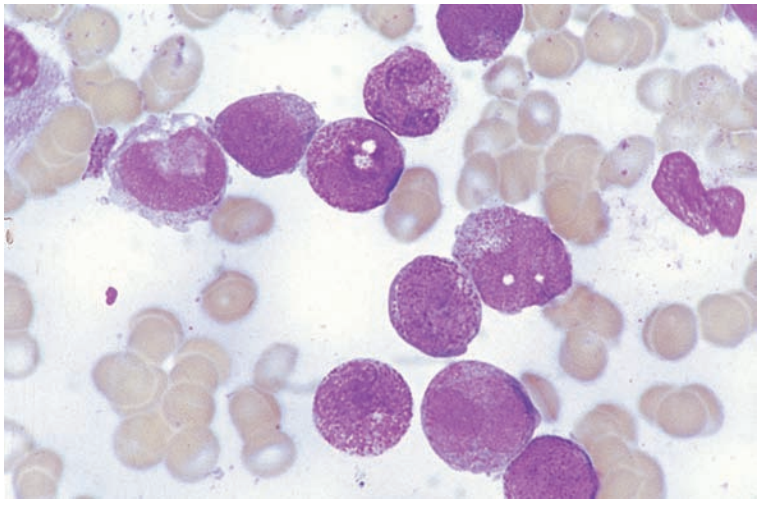


Fig. 1.16 BM film of a patient with FAB M2 AML showing unusually heavy granulation of neutrophils and precursors. MGG ×100. (By courtesy of Dr David Swirsky, Leeds.)

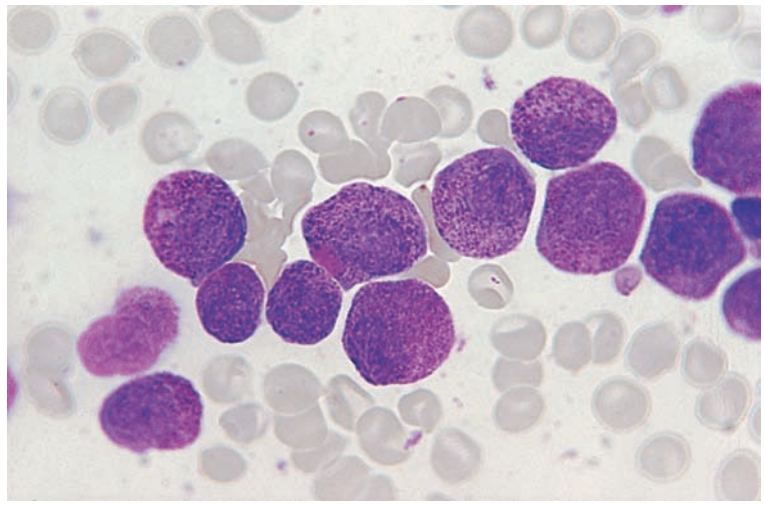


Fig. 1.17 BM film of a patient with FAB M3 AML showing hypergranular promyelocytes, one of which has a giant granule. MGG ×100.

Cytochemical reactions in M2 acute myeloid leukaemia
The cytochemical reactions in M2 AML are the same as those in M1 AML, but generally reactions are stronger and a higher percentage of cells are positive with MPO and SBB stains. CAE is more often positive in M2 than in M1 AML and reactions are stronger. Auer rods show the same staining characteristics as in M1 AML but are more numerous. When leukaemic myeloblasts undergo maturation, as occurs in M2 AML, there may be a population of neutrophils, presumably derived from leukaemic blasts that lack SBB and MPO activity. This may be demonstrated cytochemically or by means of an automated differential counter based on the peroxidase reaction, which shows a low mean peroxidase score and an abnormally placed neutrophil cluster. The neutrophil cluster with such automated instruments is often dispersed in AML in contrast to the normal compact cluster in ALL. The neutrophil alkaline phosphatase (NAP) score is often low in M2 AML.

Acute hypergranular promyelocytic leukaemia: M3 acute myeloid leukaemia
In acute hypergranular promyelocytic leukaemia, the predominant cell is a highly abnormal promyelocyte. In the majority of cases, blasts are fewer than 30% of bone marrow nucleated cells. The distinctive cytological features are sufficient to permit a diagnosis and cases are classified as M3 AML despite the low blast percentage. M3 AML is associated with a specific cytogenetic abnormality, t(15;17)(q22;q12) (see page 125), and with abnormal coagulation. There is disseminated intravascular coagulation and activation of fibrinolysis, resulting in abnormal bleeding and bruising. This diagnosis can sometimes be suspected from the prominent haemorrhagic manifestations. Typical cytological and histological features are shown in Figs 1.17–1.19. The predominant cell is a promyelocyte, the cytoplasm of which is densely packed with coarse red or purple granules, which almost obscure the nucleus. There is often nucleocytoplasmic asynchrony with the nucleus having a diffuse chromatin pattern and one or more nucleoli. When the nuclear shape can be discerned it is found, in the majority of cases, to be reniform or folded or bilobed with only a narrow bridge between the two lobes. The nuclear form is often more apparent on histological sections (Fig. 1.19). Auer rods are common. In one series they were noted in fewer than 50% of cases [39] but others have observed them to be almost always present, at least in a minority of cells [40]. In some cases there are giant granules or multiple Auer rods, which are often present in sheaves or 'faggots' (Fig. 1.18). Most cases have a minority of cells that are agranular, have sparse granules or have fine red or rust-coloured dust-like granules rather than coarse, brightly staining granules. Cells that lack granules but have lakes of hyaline pink material in the cytoplasm may also be seen. There may be basophilic differentiation in M3 AML, in addition to the

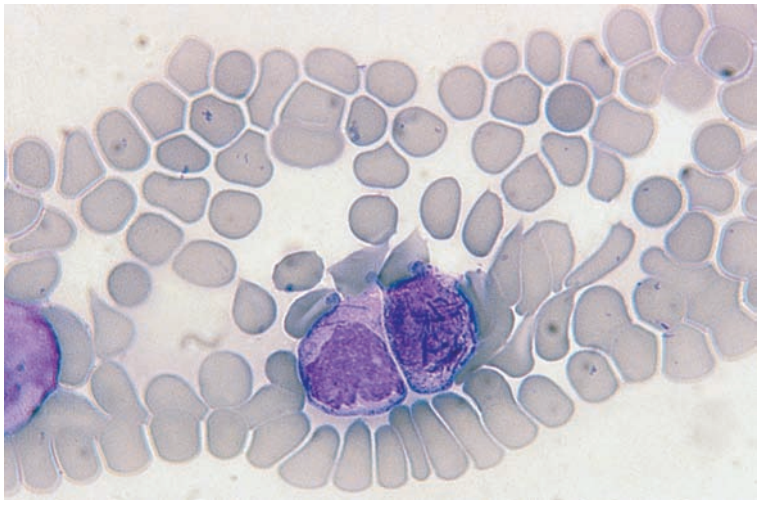


Fig. 1.18 PB film of a patient with FAB M3 AML. One of the abnormal promyelocytes contains loose bundles of Auer rods. MGG ×100.

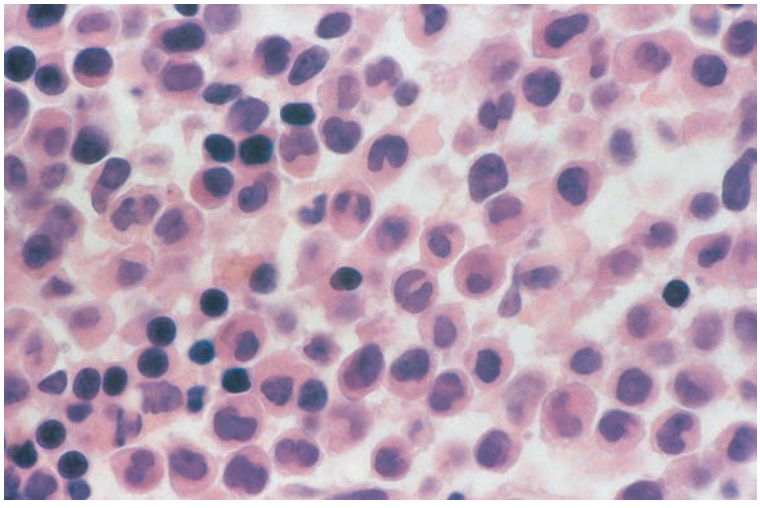


Fig. 1.19 Trephine biopsy section from a patient with FAB M3 AML. Paraffin embedded, H&E ×100.

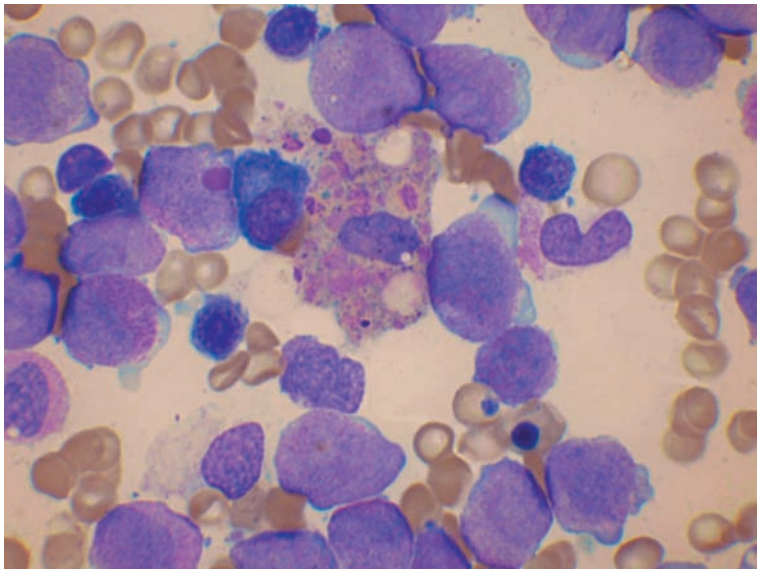


Fig. 1.20 BM film from a patient with acute promyelocytic leukaemia a few days after starting treatment with all-*trans*-retinoic acid (ATRA): one leukaemic cell contains a giant granule; a prominent macrophage contains granules and Auer rods from ingested leukaemic cells. MGG ×100.

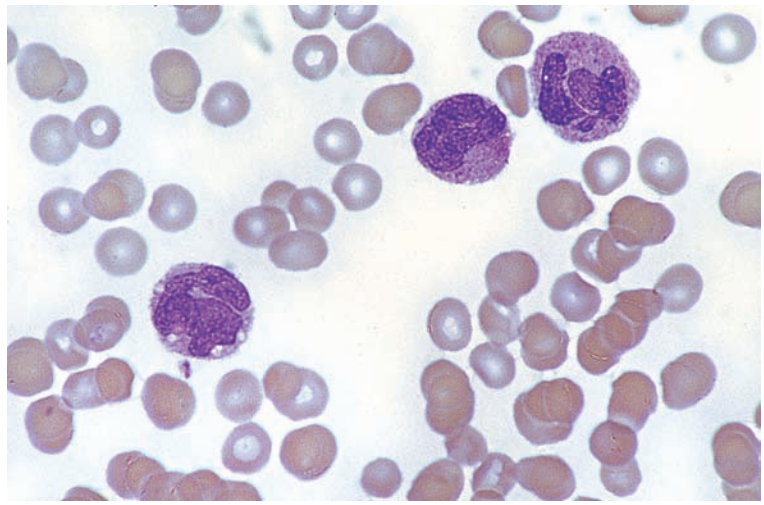


Fig. 1.21 PB film of a patient with FAB M3 AML being treated with ATRA and granulocyte colony-stimulating factor (G-CSF); leukaemic promyelocytes are undergoing maturation into highly abnormal cells. MGG ×100.

dominant neutrophilic differentiation. Bone marrow macrophages may contain giant granules or Auer rods derived from ingested leukaemic cells (Fig. 1.20). Auer rods can persist in macrophages after the patient has entered complete remission [41]. Dysplastic changes in the erythroid and megakaryocyte lineages are usually absent.

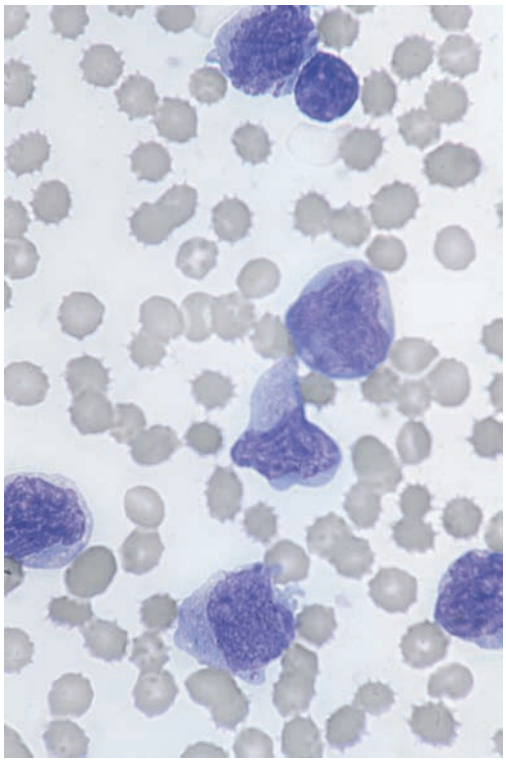
Examining an adequate bone marrow aspirate is particularly important in M3 AML, as the WBC is often low and, even when there is a leucocytosis, typical hypergranular promyelocytes may not be present in the blood. The specimen may clot during attempted aspiration, as a consequence of the associated hypercoagulable state, but usually sufficient cells are obtained for diagnosis.

M3 AML has been found to be very sensitive to the differentiating capacity of all-*trans*-retinoic acid (ATRA). Following such therapy an increasing proportion of cells beyond the promyelocyte stage are apparent. Maturing cells are cytologically abnormal (Fig. 1.21). Metamyelocytes and neutrophils may contain Auer rods. The neutrophil count rises and in some patients also the basophil count [42]. Following treatment with ATRA, the terminally differentiated cells in a late stage of apoptosis are engulfed by bone marrow macrophages [43]. M3 AML is also responsive to treatment with arsenic trioxide, As_2O_3. Hyperleucocytosis may occur during therapy with both ATRA and arsenic trioxide [44].

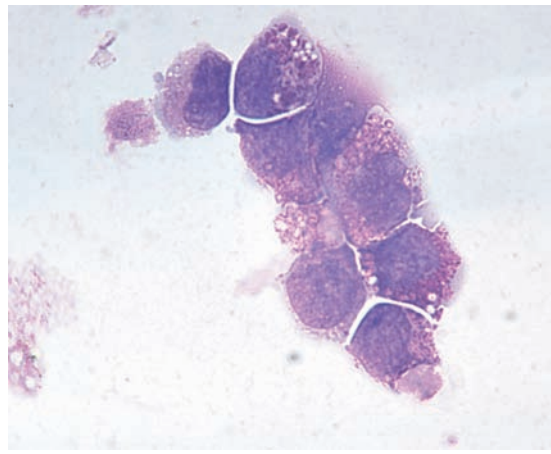
The variant form of acute promyelocytic leukaemia: M3 variant acute myeloid leukaemia

Some years after the initial description of hypergranular promyelocytic leukaemia it was noted that there were other cases of acute leukaemia that showed the same cytogenetic abnormality and coagulation abnormality but were cytologically different. Such cases were recognized as a variant form of promyelocytic leukaemia, designated microgranular or hypogranular promyelocytic leukaemia [45–47]. Such cases were subsequently incorporated into the FAB classification as M3 variant (M3V) AML. In addition to cytogenetic and molecular evidence indicating the close relationship of M3 and M3V AML, it has been noted that the cells of M3V may show a marked increase in granularity on culture [47] (Fig. 1.22) and, conversely, cases of M3 AML may have less granular cells on relapse [22]. There is not a clear demarcation between cases of classical M3 AML and the variant form – cases with intermediate features are seen. This is not surprising since these are morphological variants of a single biological entity.

Most cases of M3V AML are characterized by a cell with a reniform, bilobed, multilobed or convoluted nucleus and either sparse fine granules or apparently agranular cytoplasm (Fig. 1.23). A variable proportion of cells may have multiple Auer rods, fine dust-like granules, or large oval, elliptiform or somewhat angular cytoplasmic inclusions with

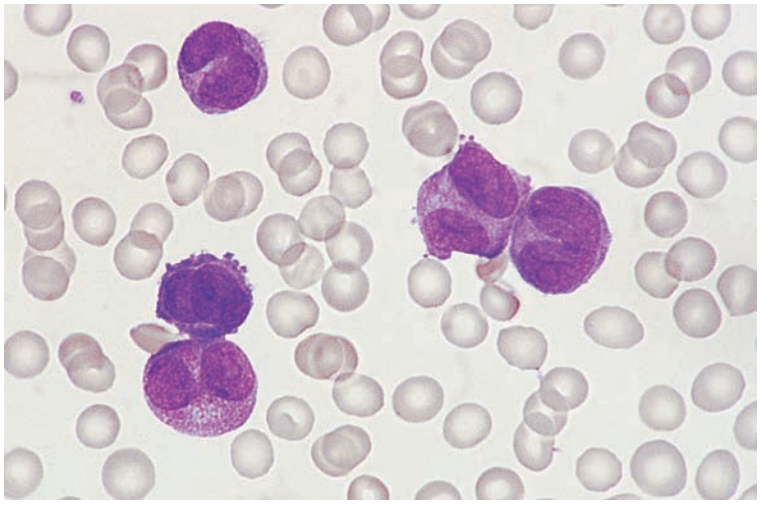


(a)



(b)

Fig. 1.22 (a) PB film and (b) film of cultured leukaemic cells from a patient with FAB M3 variant AML showing the acquisition of granules on culture. MGG ×100. (By courtesy of Professor David Grimwade, London.)

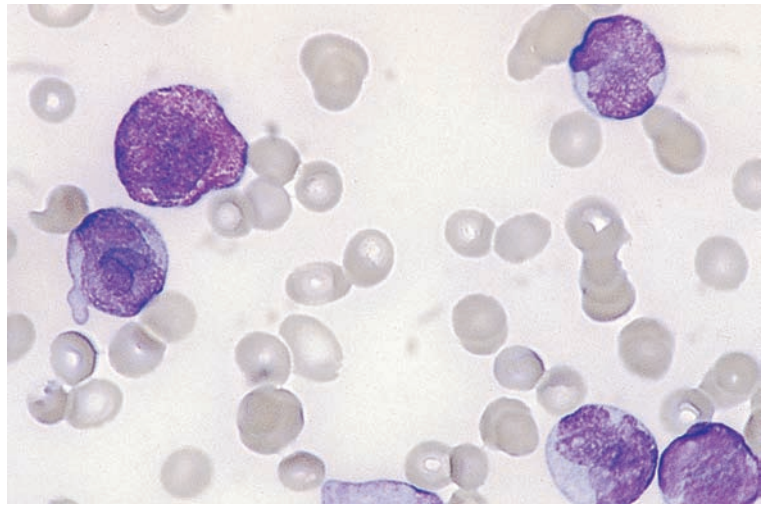


(a)

Fig. 1.23 (a) PB film of a patient with FAB M3 variant AML showing cells with bilobed and reniform nuclei and sparse, fine granules. One binucleate cell is present and another cell is characterized by basophilic cytoplasm and cytoplasmic projections. MGG ×100.

the same staining characteristics as primary granules. Typical hypergranular promyelocytes constitute a small minority of the leukaemic cells in the peripheral blood but they are usually more numerous in the bone marrow. On ultrastructural examination, the granules are smaller and usually less numerous than in hypergranular promyelocytic leukaemia [22] (Fig. 1.24). The WBC is usually higher in M3V than in M3 AML.

In a minority of cases of M3V AML the characteristic cell is a small, abnormal promyelocyte with the same lobulated nucleus as described above but with hyperbasophilic cytoplasm; cytoplasmic projections are sometimes present so that cells may resemble



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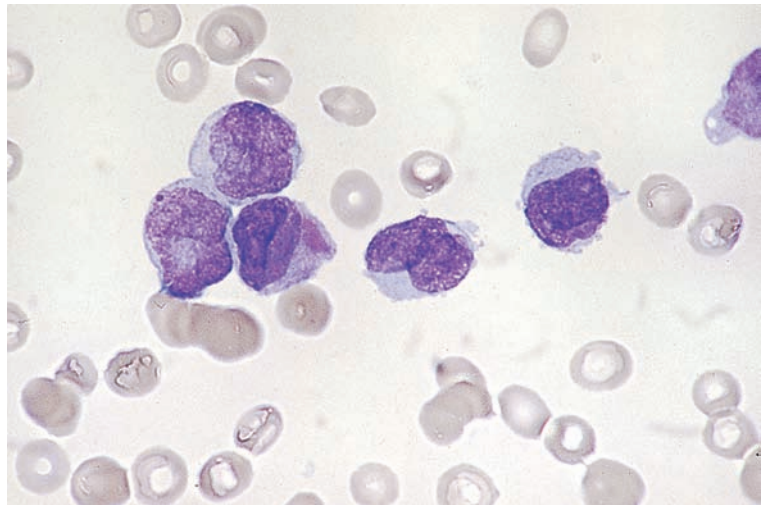
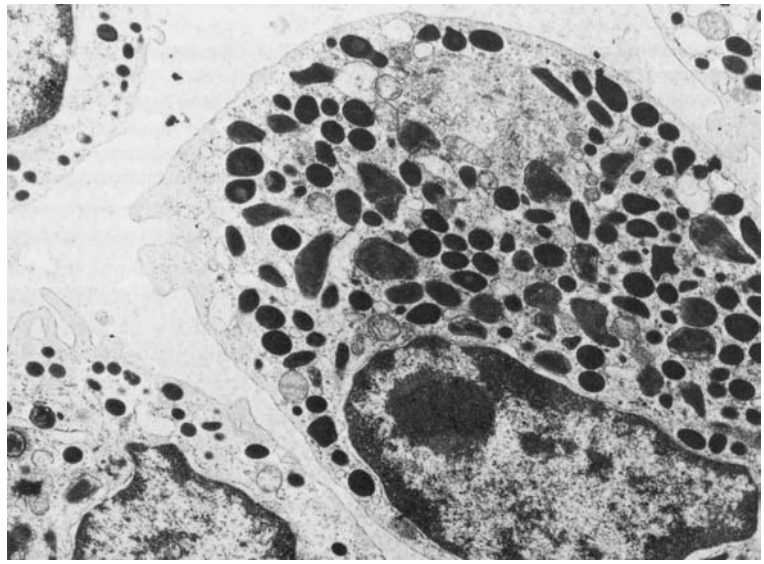


(c)

Fig. 1.23 (*Continued*) (b and c) PB film of another patient with FAB M3 variant AML showing: (b) predominantly agranular cells with twisted nuclei but with one typical hypergranular cell being present; (c) agranular cells with twisted nuclei; one cell contains a large azurophilic inclusion. MGG ×100.

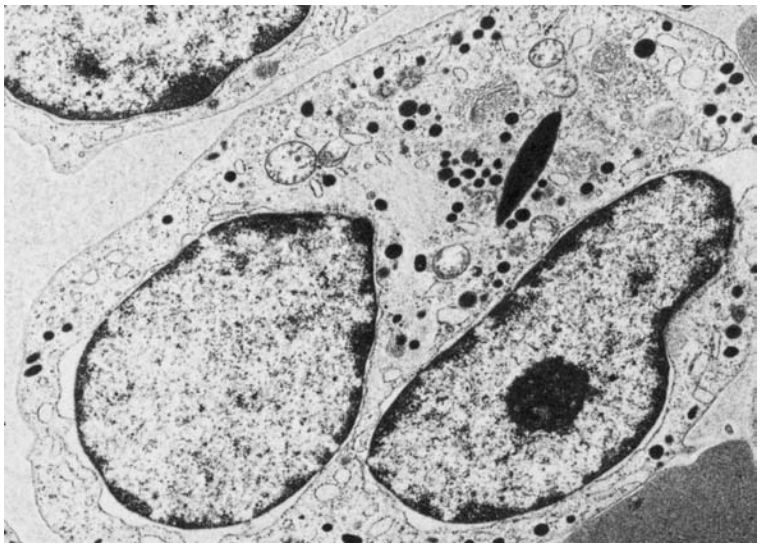


(a)

Fig. 1.24 Ultrastructural examination of leukaemic cells of three patients with acute promyelocytic leukaemia: (a) typical M3 AML showing granules that are numerous and large.

Continued

(b)



(c)

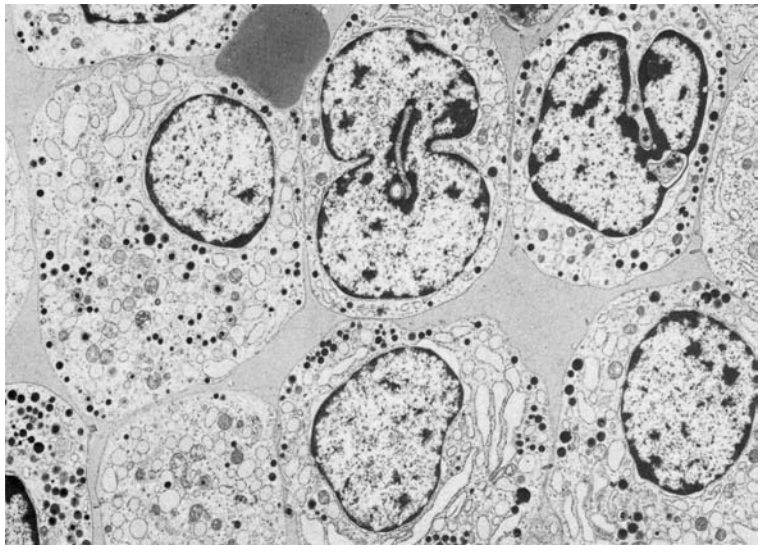


Fig. 1.24 (*Continued*) (b) M3 variant AML showing a lobulated nucleus, granules that are smaller and more sparse and one Auer rod; (c) hyperbasophilic M3 variant AML showing fewer and smaller granules than in typical M3 AML plus abundant dilated rough endoplasmic reticulum. Uranyl acetate, lead citrate stain. (By courtesy of Dr Robert McKenna, Minnesota and the British Journal of Haematology.)

megakaryoblasts [22] (Fig. 1.25). Such cells are seen in the majority of cases of M3V AML, but usually as a minor population. On ultrastructural examination, there are sparse small granules and, in addition, abundant dilated rough endoplasmic reticulum [22] (Fig. 1.24).

M3V may be confused with acute monocytic leukaemia (M5b) if blood and bone marrow cells are not examined carefully and if the diagnosis is not considered. The use of an automated blood cell counter based on cytochemistry (MPO or SBB) is useful for the rapid distinction between M3V and M5 AML (see Fig. 1.71). When M3V appears likely from the cytological and cytochemical features, the diagnosis can be confirmed by cytogenetic, molecular genetic or immunophenotypic analysis.

When treated with chemotherapy, the prognosis of M3 variant is somewhat worse than that of M3 AML [48]. This is likely to be related to the higher WBC, since the WBC is of prognostic importance in M3/M3V AML [30], and the greater prevalence of a secondary mutation, an internal tandem duplication

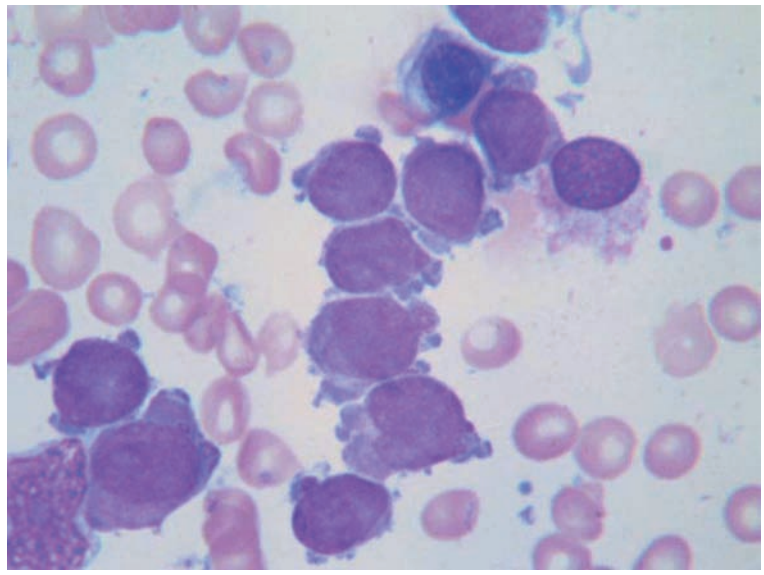


Fig. 1.25 BM film from a patient with the hyperbasophilic variant of acute promyelocytic leukaemia showing abnormal promyelocytes with blebbed basophilic cytoplasm; some also have a dusting of fine cytoplasmic granules. MGG ×100.

of the *FLT3* gene (*FLT3*-ITD). As a higher WBC remains an adverse prognostic feature when M3/M3V AML is treated with ATRA plus chemotherapy [48] it is likely that M3V also has a worse prognosis with combined modality treatment.

M3 and M3V AML usually together constitute 5–10% of cases of AML but in Hispanics promyelocytic leukaemia is more frequent.

Cytochemical reactions in M3 and M3 variant acute myeloid leukaemia Hypergranular promyelocytes are usually strongly positive with MPO, SBB and CAE stains although cases have been described that were MPO and SBB negative but were strongly CAE positive [49]. The PAS reaction usually shows a cytoplasmic 'blush' – a fine diffuse or dust-like positivity; the reaction is stronger than in M1 or M2 AML. PAS-positive erythroblasts are not generally seen. The acid phosphatase reaction is strongly positive. M3V AML usually shows similar cytochemical reactions [22] (Fig. 1.26) but sometimes the reactions are weaker [50]. A potentially confusing cytochemical reaction in both M3 and M3V AML is the presence in some cases of NSE activity [21,22,39], a reactivity otherwise characteristic of monocytic rather than granulocytic differentiation. ANAE, ANBE and NASDA may be positive and, as for the monocytic lineage, the reaction is fluoride sensitive. The reaction is weaker than in monocytes, and isoenzymes characteristic of the monocytic lineage are not present [50]. Some cells show double staining for NSE and CAE. Cases that are positive for ANAE tend to have a weaker reaction for CAE, and occasionally the MPO reaction is unexpectedly weak [39]. The minority of cases that are positive for NSE do not appear to differ from other cases with regard to morphology, haematological or cytogenetic findings or prognosis [39].

Cases with basophilic differentiation show metachromatic staining with toluidine blue.

Auer rods in M3 AML are SBB, MPO and CAE positive, whereas in other categories of AML they are usually negative with CAE; they may be weakly PAS positive. On SBB, MPO and CAE staining, the core of the rod may be left unstained and occasionally the core is ANAE positive on a mixed esterase stain [8].

Acute myelomonocytic leukaemia: M4 acute myeloid leukaemia

The criteria for the diagnosis of AML of M4 subtype, that is, AML with both granulocytic and monocytic differentiation, are shown in Table 1.7 and typical cytological and histological features in Figs 1.27 and 1.28. The criterion for recognition of a significant granulocytic component is a morphological one; the granulocytic component, which in this context includes myeloblasts as well as maturing cells, must

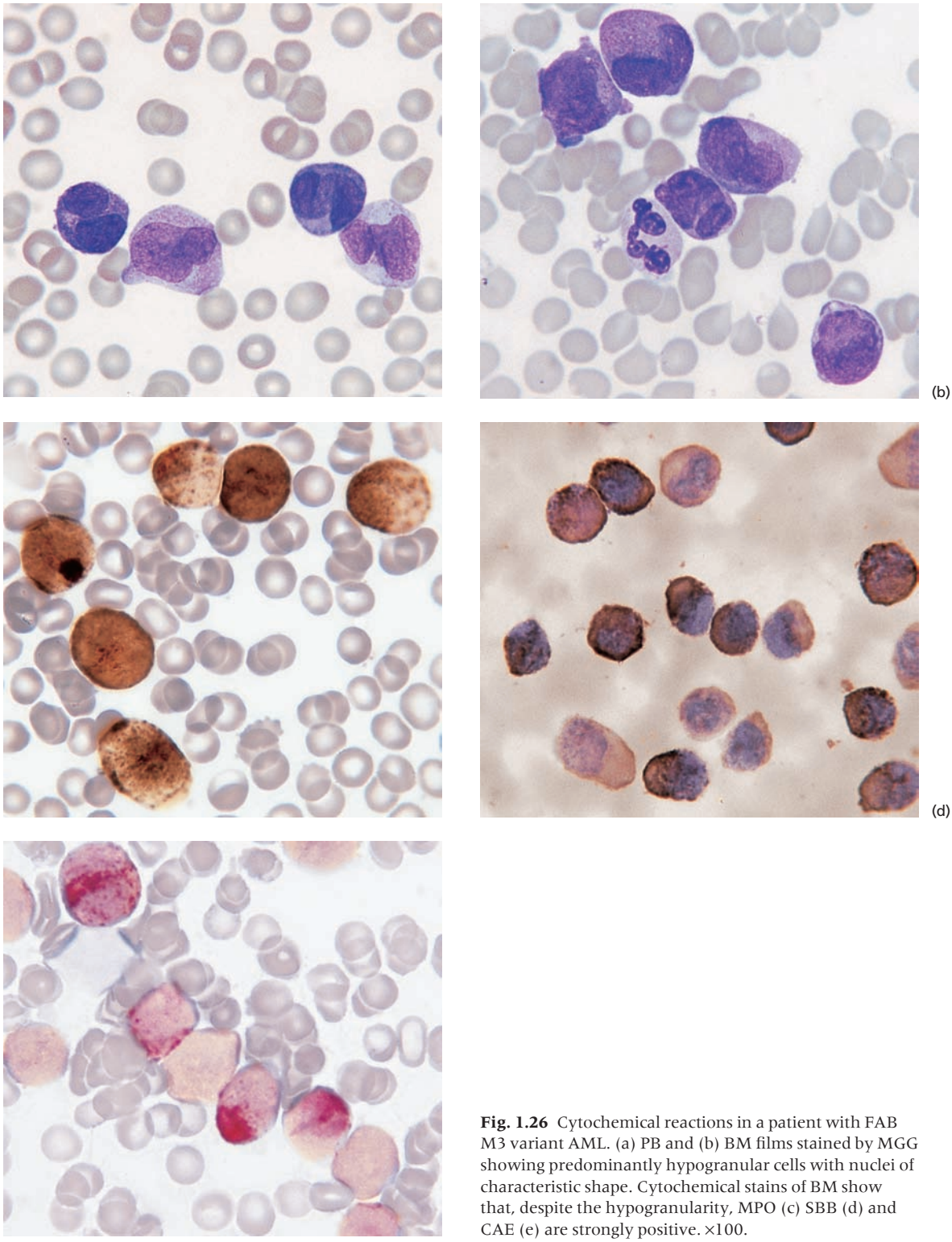
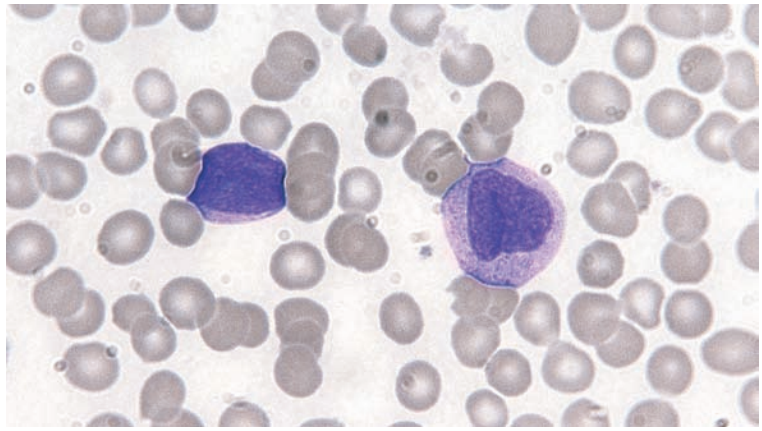


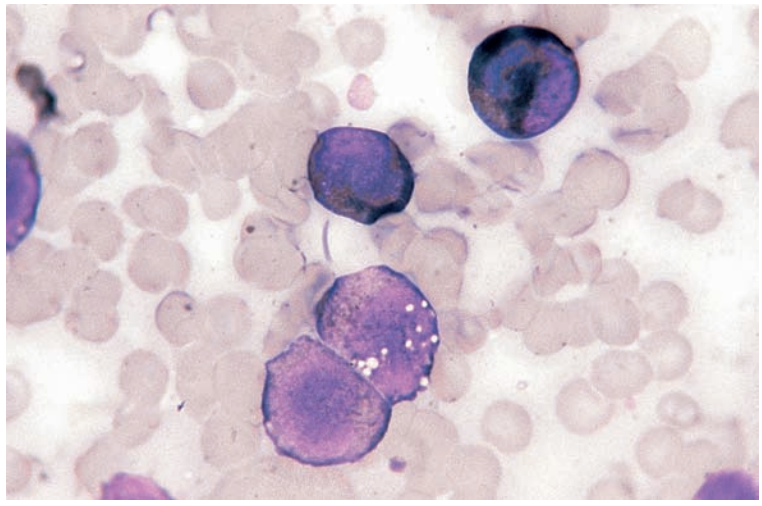
Fig. 1.26 Cytochemical reactions in a patient with FAB M3 variant AML. (a) PB and (b) BM films stained by MGG showing predominantly hypogranular cells with nuclei of characteristic shape. Cytochemical stains of BM show that, despite the hypogranularity, MPO (c) SBB (d) and CAE (e) are strongly positive. ×100.

Table 1.7 Criteria for the diagnosis of acute myeloid leukaemia of M4 category (acute myelomonocytic leukaemia).

- Blasts ≥30% of bone marrow cells
- Blasts ≥30% of bone marrow non-erythroid cells
- Bone marrow granulocytic component (myeloblasts to polymorphonuclear leucocytes) ≥20% of non-erythroid cells
- Significant monocytic component as shown by one of the following:
  - bone marrow monocytic component (monoblasts to monocytes) ≥20% of non-erythroid cells and peripheral blood monocytic component ≥5×10^9/l, *or*
  - bone marrow monocytic component (monoblasts to monocytes) ≥20% of non-erythroid cells and confirmed by cytochemistry or increased serum or urinary lysozyme concentration, *or*
  - bone marrow resembling M2 but peripheral blood monocyte component ≥5×10^9/l and confirmed by cytochemistry or increased serum or urinary lysozyme concentration



(a)



(b)

Fig. 1.27 (a) PB film of a patient with FAB M4 AML showing a myeloblast which is of medium size with a high nucleocytoplasmic ratio and a monoblast which is larger with more plentiful cytoplasm and a folded nucleus with a lacy chromatin pattern. MGG ×100. (b) BM of the same patient stained with SBB showing two monoblasts with a weak granular reaction and two cells of the granulocytic series with a much stronger reaction. SBB ×100.

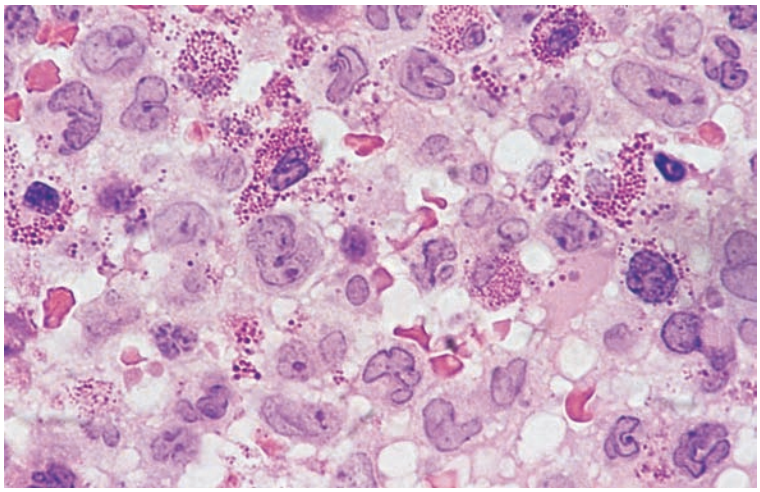


Fig. 1.28 Trephine biopsy section from a patient with M4Eo AML. Cells are either monoblasts/promonocytes, recognized as large cells with round or lobulated nuclei containing prominent nucleoli, or eosinophils. Resin embedded, H&E ×100.

be at least 20% of non-erythroid cells. The recognition of a significant monocytic component requires two criteria to be satisfied, which may be both morphological or one morphological and the other cytochemical, as shown in Table 1.7. In assessing the monocytic component, monoblasts, promonocytes and monocytes are included in the count.

The FAB criteria for the recognition of monocytic differentiation are the presence of fluoride-sensitive naphthol AS acetate esterase (NASA) or NASDA activity [1], or the presence of ANAE activity [4]. ANBE activity would also identify monocytic differentiation. Alternatively, lysozyme activity of leukaemic cells can be demonstrated cytochemically or lysozyme concentration can be measured in serum or urine, an elevation to more than three times the normal value being regarded as significant [4]. Careful examination of the peripheral blood is important if all cases of M4 AML are to be recognized since the bone marrow is sometimes morphologically indistinguishable from that of M2 AML. In M4 AML, the granulocytic differentiation is usually along the neutrophil pathway, but in some cases it is eosinophilic (M4Eo) (Fig. 1.28), basophilic (M4Baso) or both (Fig. 1.29).

The M4 subtype accounts for 15–20% of cases of AML.

Cytochemical reactions in M4 acute myeloid leukaemia
In M4 AML some leukaemic cells show cytochemical reactions typical of neutrophilic, eosinophilic or basophilic lineages while other cells show reactions typical of the monocytic lineage (see above). A double esterase stain for CAE (neutrophil lineage) and ANAE (monocyte lineage) [51] is a convenient method for demonstrating the pattern of differentiation and maturation in M4 AML (Fig. 1.29c).

Acute monocytic/monoblastic leukaemia: M5 acute myeloid leukaemia

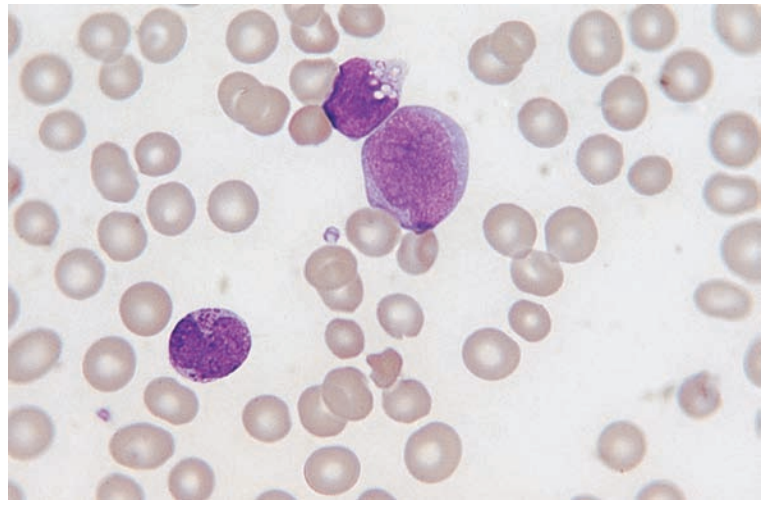
The criteria for the diagnosis of acute monocytic/monoblastic leukaemia, M5 AML, are shown in Table 1.8, and typical cytological and histological features in Figs 1.30–1.33. This diagnosis may be suspected from clinical features when there is infiltration of the skin and the gums. Disseminated intravascular coagulation and increased fibrinolysis are more common in M5 AML than in other categories of AML, with the exception of M3 [52].

Table 1.8 Criteria for the diagnosis of acute myeloid leukaemia of M5 category (acute monoblastic/monocytic leukaemia).

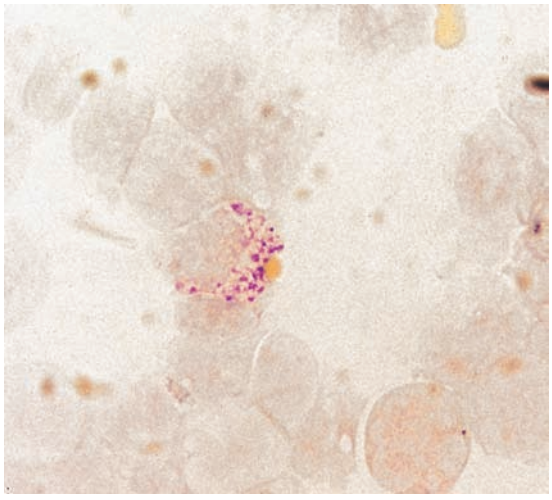
- Blasts ≥30% of bone marrow cells
- Blasts ≥30% of bone marrow non-erythroid cells
- Bone marrow monocytic component ≥80% of non-erythroid cells

Acute monoblastic leukaemia (M5a)
- Monoblasts ≥80% of bone marrow monocytic component

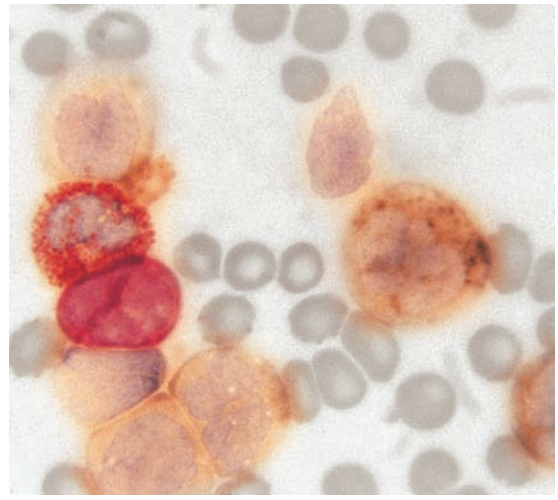
Acute monocytic leukaemia (M5b)
- Monoblasts <80% bone marrow monocytic component



(a)



(b)



(c)

Fig. 1.29 PB film of a patient with M4 AML – M4Eo/inv(16) – who had both eosinophil and basophil differentiation. (a) A blast cell and two primitive cells containing basophil granules; one of the latter is vacuolated. MGG ×100. (b) Toluidine blue stain showing metachromatic staining of a basophil precursor. ×100. (c) Double esterase stain showing positivity of the granulocyte series with CAE (red) and positivity of the monocyte series with α-naphthyl acetate (non-specific) esterase (ANAE) (brownish-black). ×100.

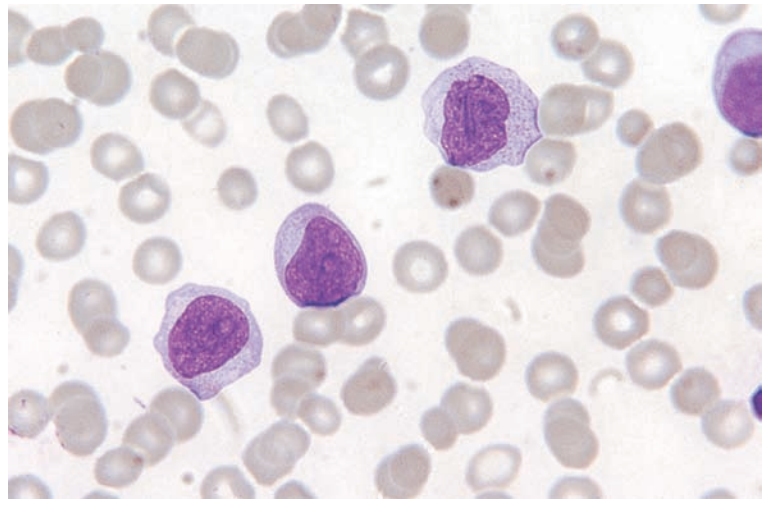


Fig. 1.30 PB film of a patient with FAB M5a AML showing three monoblasts. MGG ×100.

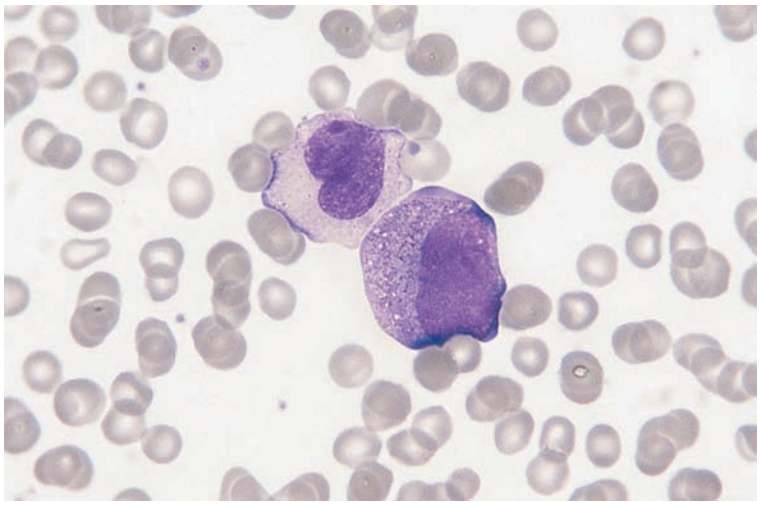
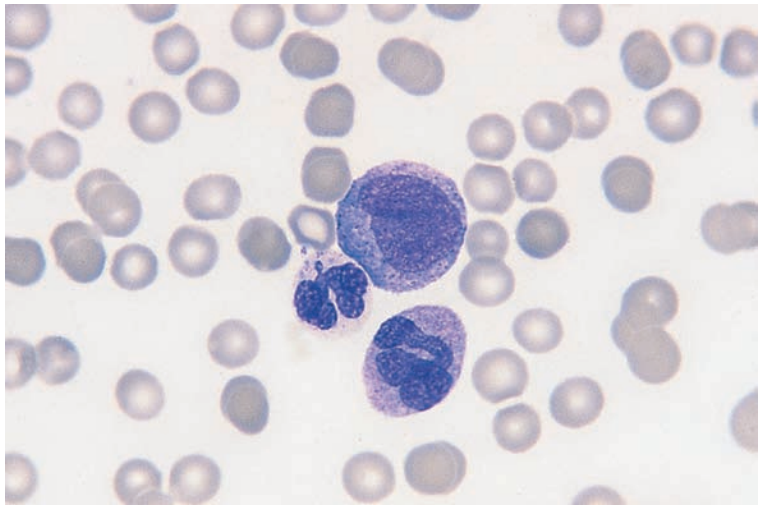


Fig. 1.31 PB film of a patient with FAB M5b AML showing a monocyte and a promonocyte; the latter is moderately heavily granulated. MGG ×100.

(a)



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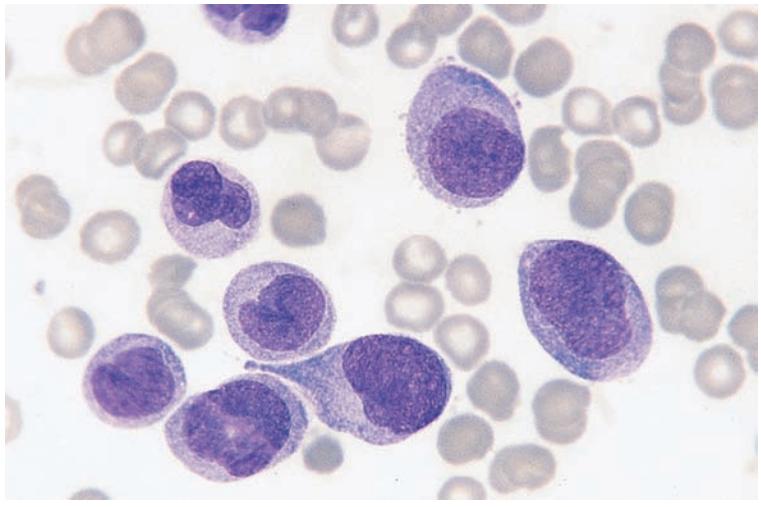


Fig. 1.32 PB and BM films of a patient with FAB M5b AML in whom the PB cells were more mature than the BM cells. (a) PB film showing a promonocyte and a monocyte with a nucleus of abnormal shape; the third cell is probably an abnormal neutrophil. MGG ×100. (b) BM film showing predominantly monoblasts and promonocytes. MGG ×100.

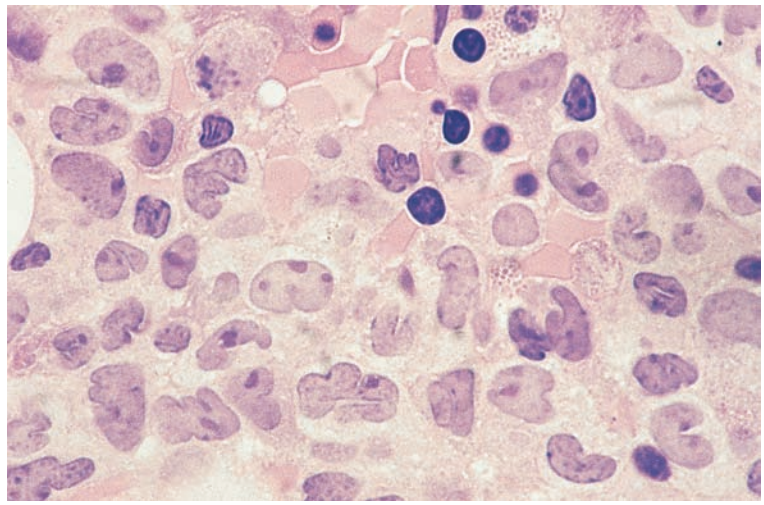


Fig. 1.33 Trephine biopsy section from a patient with FAB M5b AML and myelodysplasia. Monoblasts and monocytes can be identified; the former are the larger cells with a round nucleus, a dispersed chromatin pattern and prominent nucleoli whereas the latter have lobulated nuclei. The cells with smaller dark nuclei are erythroblasts, one of which has a nucleus of abnormal shape. Resin embedded, H&E ×100.

M5 AML is further subdivided into M5a AML (acute monoblastic leukaemia) and M5b AML (acute monocytic leukaemia) on the basis of whether monoblasts comprise at least 80% of the total bone marrow monocytic component. Auer rods are quite uncommon in M5 AML. There is often disorderly maturation leading to cells of monocytic lineage with nucleocytoplasmic asynchrony and other dysplastic features. This can make it difficult to assign cells reliably to monoblast, promonocyte and immature monocyte categories. Leukaemic cells in the peripheral blood may be more mature than those in the bone marrow (Fig. 1.32). Monocytic differentiation can be confirmed by cytochemistry and by measurement of urinary and serum lysozyme concentrations; immunophenotyping can also be helpful.

In a rare form of acute monocytic leukaemia, cells have cytological features resembling those of macrophages or histiocytes (Fig. 1.34). This may be regarded as the leukaemic phase of malignant histiocytosis. The designation M5c has been suggested [53].

M5a and M5b are cytogenetically and genetically distinct. M5a has a significantly higher prevalence of cytogenetic abnormalities with an 11q23 breakpoint and *MLL* rearrangement, and also of isolated trisomy 8 [54]; it has a lower prevalence of *FLT3*-ITD.

There is some evidence that M5 AML is more sensitive than other types of AML to certain antileukaemic drugs, specifically etoposide, cytarabine, anthracyclines, vincristine, asparaginase and cladribine [55].

The M5 subtype accounts for about 15% of cases of AML.

Cytochemical reactions in M5 acute myeloid leukaemia
In M5a AML, MPO and SBB reactions are often negative, although a few fine, positive granules may be present. CAE is negative or very weak. Hayhoe and Quaglino [8] found SBB to be more sensitive than MPO in detecting monocytic differentiation; they noted that, with SBB, granules in monoblasts were usually scattered and fine whereas in myeloblasts the reaction was either localized or filled all the cytoplasm. Monoblasts were characteristically negative for MPO. Monoblasts are usually strongly positive for NSE, i.e. for ANAE (Fig. 1.35a), ANBE, NASA (Fig. 1.35b) and NASDA. All these esterase activities are inhibited by fluoride but only in the case of NASA and NASDA is it necessary to carry out the reaction with and without fluoride to convey specificity; in the case of ANAE and ANBE, the reaction is negative or weak in cells of the granulocytic lineage. Aberrant esterase reactions are sometimes seen; occasional cases have negative reactions for NSE and other cases, when the reaction for NSE is very strong, give a positive reaction also for CAE. Monoblasts show diffuse acid phosphatase activity, which, along with NSE activity, appears in advance of SBB and MPO reactivity. Lysozyme activity, which appears at about the same

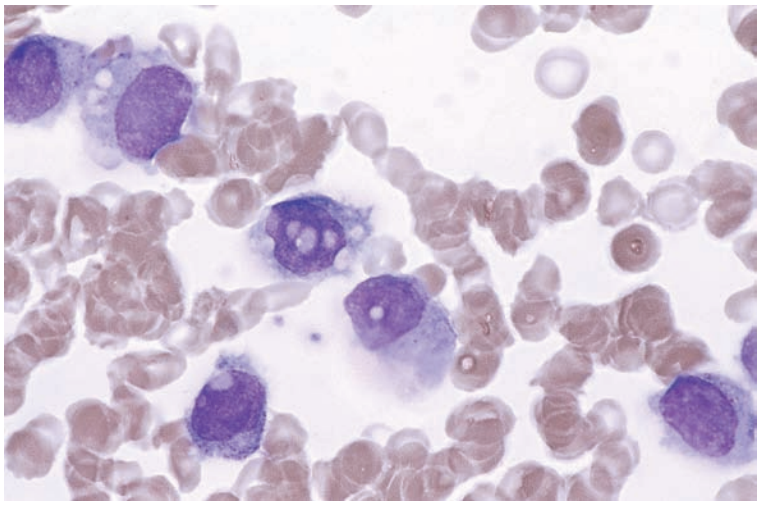
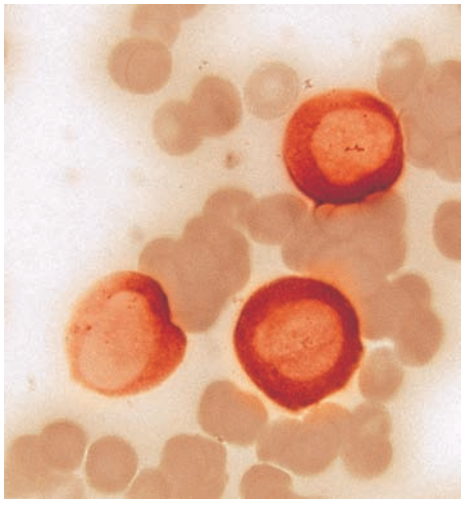
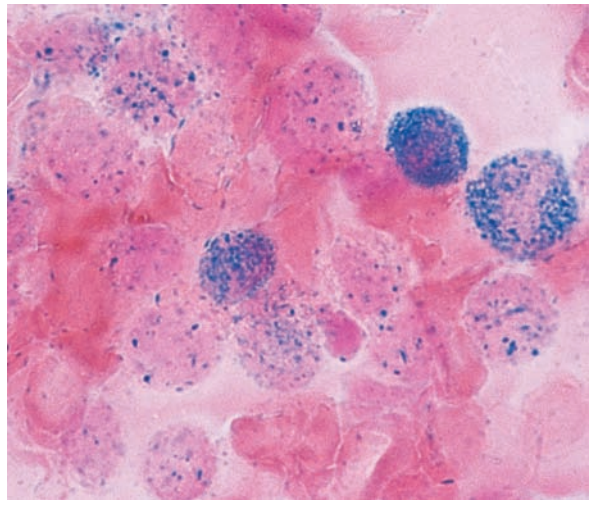


Fig. 1.34 BM film of a patient with leukaemic cells showing histiocytic or macrophage differentiation ('M5c' AML). MGG ×100.



(a)



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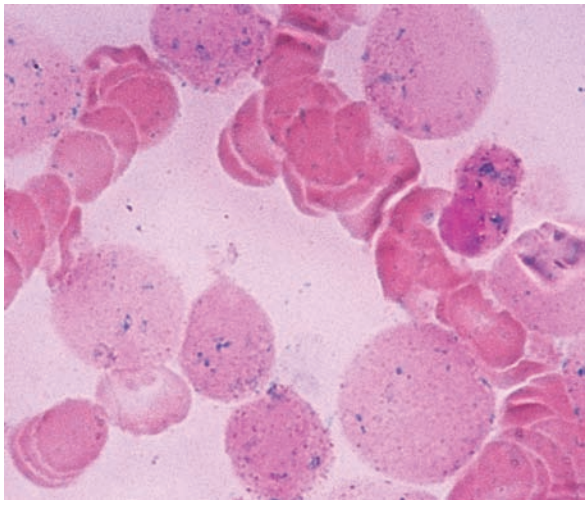
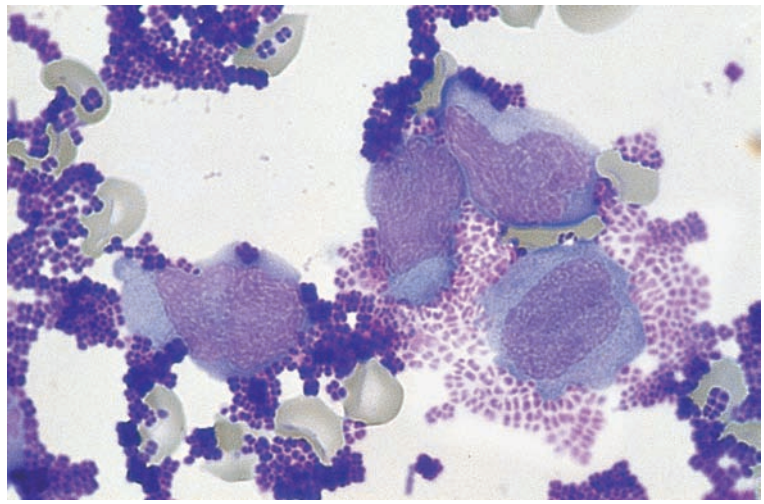
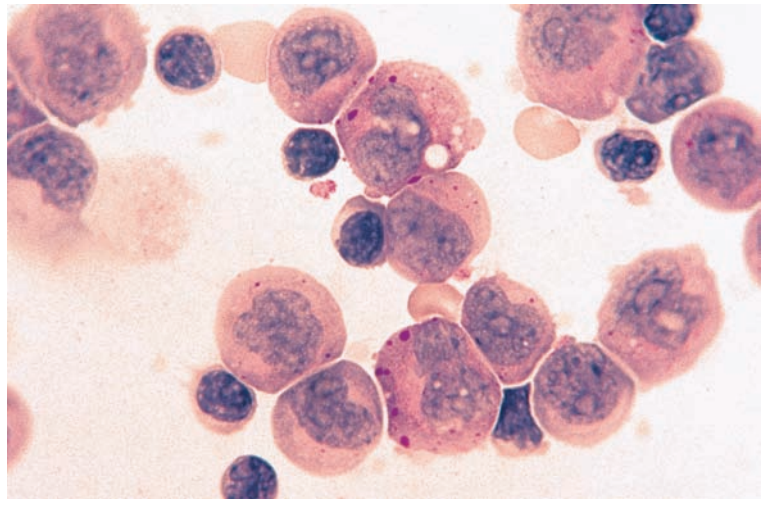


(c)

Fig. 1.35 (a) BM film of a patient with FAB M5a AML stained for ANAE activity. ANAE ×100. (b, c) BM film of a patient with FAB M5b AML stained for naphthol AS acetate esterase (NASA) activity without (b) and with (c) fluoride; inhibition of activity by fluoride is apparent. NASA ×100.



(d)



(e)

Fig. 1.35 (*Continued*) (d) Lysozyme preparation from a patient with FAB M5b AML. Leukaemic cells have been mixed with a suspension of *Micrococcus lysodeikticus* bacteria; some of the leukaemic cells have secreted lysozyme, which has lysed adjacent bacteria so that they appear paler in comparison with intact bacteria (same patient as b and c). This test is now only of historic interest. MGG ×100. (e) Periodic acid–Schiff (PAS) stain of a PB cytospin preparation from a patient with FAB M5a AML showing block positivity superimposed on fine granular and diffuse positivity. PAS ×100.

time as MPO activity, can be demonstrated cytochemically (Fig. 1.35d). The PAS reaction of monoblasts is either negative or diffusely positive with a superimposed fine or coarse granular positivity or, occasionally, superimposed PAS-positive blocks (Fig. 1.35e). In M5 AML, the NAP score is usually normal or high in contrast to the low score that may be seen in cases of AML in which granulocytic maturation is occurring.

It should be noted that in some case of M5a AML there are negative reactions for SBB, MPO and NSE. Such cases will be recognized as monoblastic only if the cytological features are assessed in relation to the immunophenotype. If the FAB classification is used such cases are classified as M0 AML but an alternative approach would be to classify cases as M5a when the cytological and immunophenotypic features (and possibly associated cytogenetic abnormalities) favour the monocytic lineage.

Acute myeloid leukaemia with predominant erythroid differentiation: M6 acute myeloid leukaemia

The FAB criteria for diagnosis of M6 AML are shown in Table 1.9 and cytological and histological features in Figs 1.36–1.42. Some cases of M6 AML represent leukaemic transformation of MDS and a significant proportion of reported cases have been

Table 1.9 Criteria for the diagnosis of acute myeloid leukaemia of M6 category (acute erythroleukaemia).

- Erythroblasts ≥50% of bone marrow nucleated cells
- Blasts ≥30% of bone marrow non-erythroid cells

therapy related; however in one large series only 1 of 62 cases was therapy related with another 2 of 62 being secondary to MDS [56]. Presentation is often with pancytopenia and macrocytosis, with circulating blast cells being present in only a minority of patients [56]. Schistocytes, teardrop cells, pincer cells and basophilic stippling are often present [56]. Circulating erythroblasts are present in around half of patients [56] and may show dysplastic features. Circulating micromegakaryocytes are present in a significant minority [56]. There may also be hypogranular and hypolobated neutrophils and giant and hypogranular platelets. In the bone marrow, moderate to marked erythroid dysplasia is particularly common, with erythroid precursors showing features such as nucleocytoplasmic asynchrony (megaloblastosis), nuclear lobulation, karyorrhexis, binuclearity, internuclear bridges, basophilic stippling and cytoplasmic vacuolation.

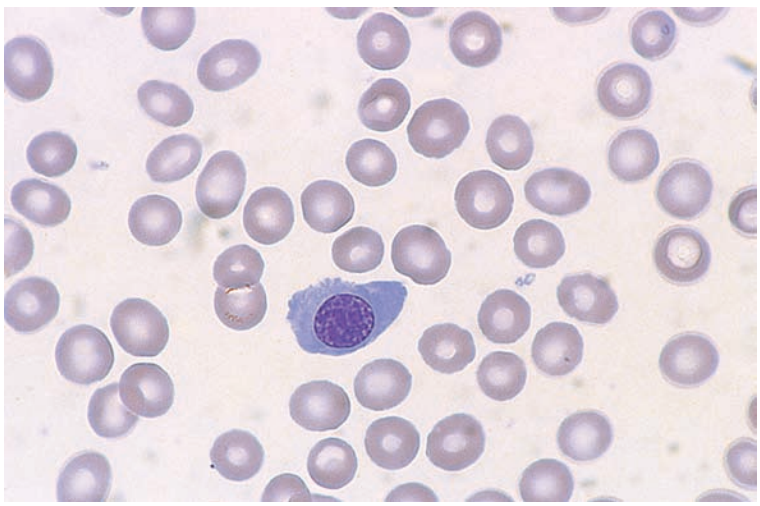


Fig. 1.36 PB film in a patient with FAB M6 AML showing anaemia, severe thrombocytopenia and an abnormal circulating erythroblast. MGG ×100.

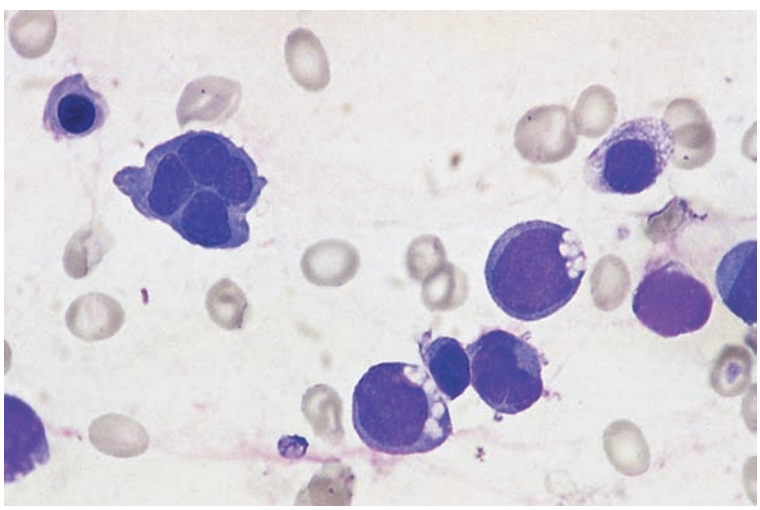


Fig. 1.37 BM film from a patient with FAB M6 AML (erythroleukaemia) showing a multinucleated erythroblast and two heavily vacuolated myeloblasts. MGG ×100.

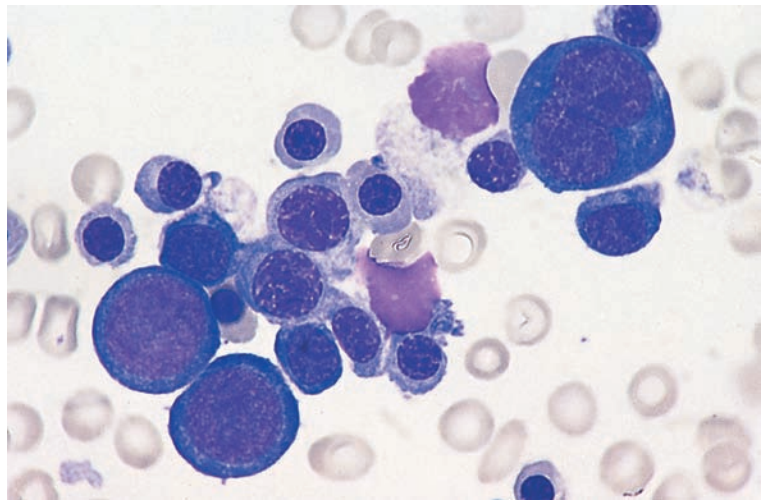
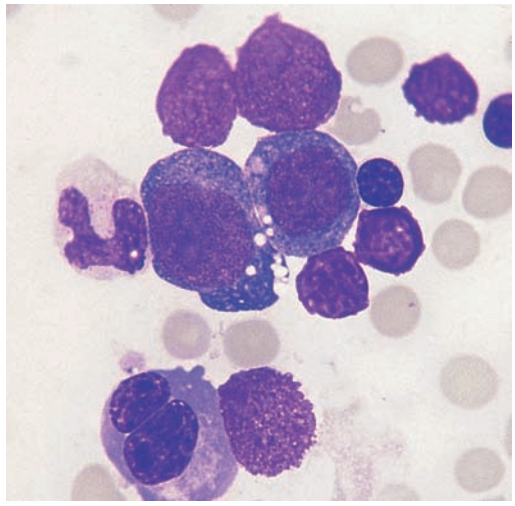
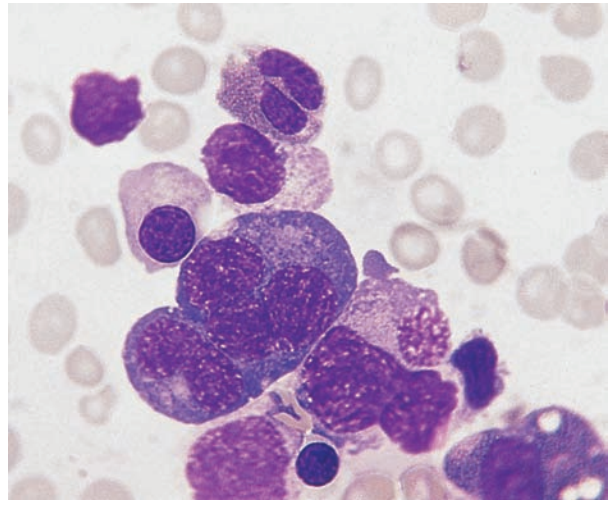


Fig. 1.38 BM film from a patient with M6 AML showing marked erythroid hyperplasia but only mild dyserythropoiesis; one binucleated erythroblast is present. MGG ×100.



(a)



(b)

Fig. 1.39 BM film from a patient with FAB M6 AML showing: (a) a binucleated erythroblast and two vacuolated erythroblasts; (b) a giant multinucleated erythroblast. MGG ×100.

There may be coalescence of prominent cytoplasmic vacuoles, this appearance correlating with the cytochemical demonstration of PAS positivity. Giant and multinucleated erythroid cells are sometimes prominent. In some cases, erythropoiesis is predominantly megaloblastic and in others it is macronormoblastic. Phagocytosis, particularly erythrophagocytosis, by abnormal erythroid precursors is sometimes seen. In some cases proerythroblasts and basophilic erythroblasts are markedly increased as a percentage of total erythroblasts. Dysplasia is not confined to the erythroid lineage, trilineage myelodysplasia being present in around half of patients [56–58]. The non-erythroid component of M6 may resemble any other FAB category with the exception of M3/M3V AML. Myeloblasts may show Auer rods.

The FAB criteria for M6 AML require that at least 50% of bone marrow nucleated cells are recognizable erythroblasts and that at least 30% of non-erythroid cells are blasts. There are also cases of AML in which the leukaemic cells appear by light

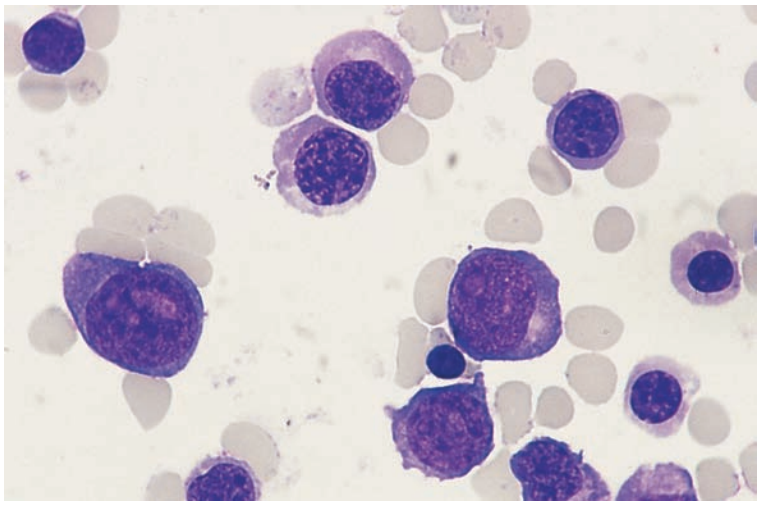
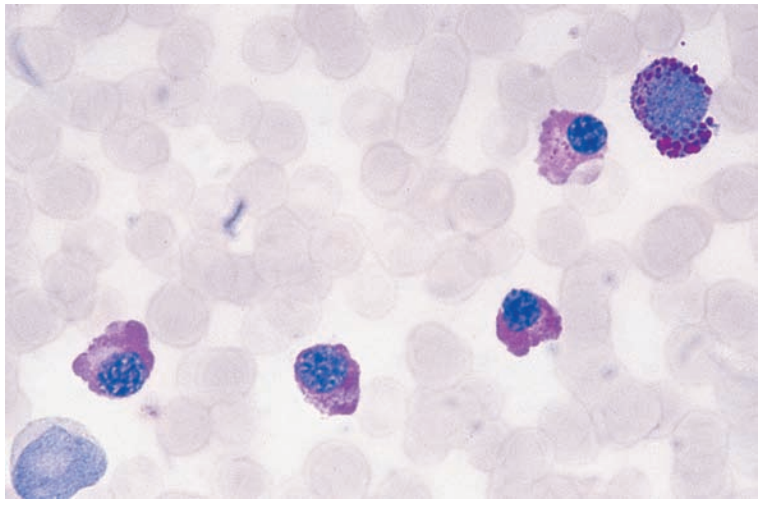


Fig. 1.40 A cytospin preparation of BM cells of a patient with FAB M6 AML showing late erythroblasts and three undifferentiated blasts. A positive reaction of the blast cells with a McAb to glycophorin A in this patient showed that these were primitive erythroid cells. MGG ×100.

(a)



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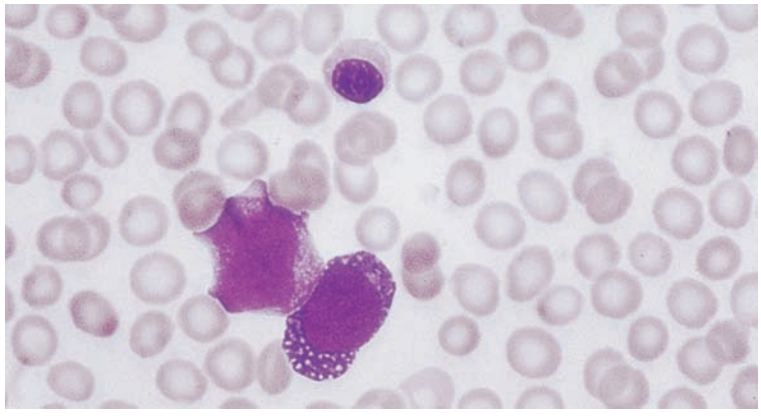


Fig. 1.41 (a, b) BM film in FAB M6 AML showing diffuse PAS positivity in late erythroblasts and block positivity in an early erythroblast; the corresponding MGG stain shows vacuolation of an early erythroblast, the vacuoles being attributable to the solubility of glycogen. (a) PAS ×100. (b) MGG ×100.

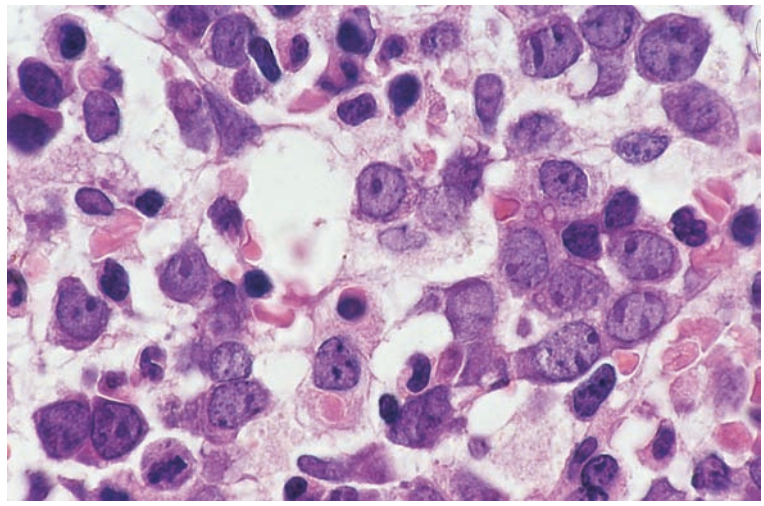


Fig. 1.42 BM trephine biopsy section in FAB M6 AML showing primitive erythroid cells, which can be distinguished from granulocyte precursors by their linear nucleoli, some of which abut on the nuclear membrane, and by their more basophilic cytoplasm (readily apparent on this Giemsa stain but not so apparent of an H&E stain). Paraffin embedded, Giemsa ×100.

microscopy to be undifferentiated blasts but can be shown by immunophenotyping or ultrastructural analysis to be primitive erythroid cells. When such cases lack a significant non-erythroid component, including more than 30% of non-erythroid blasts, they do not fit the FAB criteria for M6 AML. Nevertheless, it seems reasonable that such cases (which are rare except when AML occurs in Down syndrome [59]) should be assigned to the M6 category. Domingo-Claros *et al.* [56] found only 2 of 62 cases of erythroleukaemia to have these characteristics. Use of the terms M6 variant or 'pure erythraemia' is appropriate [56,58,60].

Overall the M6 category accounts for about 3–4% of cases of AML. The frequency is higher in the elderly [61]. Prognosis appears to be worse than for AML in general [30,62]. The survival of patients with M6 variant AML was a great deal worse than the survival of patients with FAB M6 AML in one series of patients [57], but in another both had an equally bad prognosis [58].

Cytogenetic abnormalities in M6 AML differ significantly from those in other types of AML. Cytogenetic abnormalities are common and include those of adverse prognostic significance; there may be complex cytogenetic abnormalities, hypodiploidy and abnormalities of chromosomes 5, 7, 8 and 17 [57,63].

Cytochemical reactions in M6 acute myeloid leukaemia

In M6 AML, myeloblasts and any Auer rods show the same cytochemical reactions as in other categories of AML. The NAP score may be reduced or increased and a population of neutrophils lacking SBB and MPO activity may be present.

On a PAS stain the erythroblasts show diffuse or finely granular positivity with or without coarse granular or block positivity (see Fig. 1.41). Hayhoe and Quaglino [8] have described a characteristic block or granular positivity in early erythroblasts and diffuse positivity in late erythroblasts and some erythrocytes. PAS positivity is not pathognomonic of M6 AML, being seen also in iron deficiency anaemia, severe haemolytic anaemia and thalassaemia major and in occasional cases of megaloblastic anaemia. PAS positivity is seen also in MDS and in other categories of AML (overall in about one in five cases) and suggests that the erythroblasts, even when they are fewer than 50% of nucleated cells, are part of the leukaemic or myelodysplastic clone. Erythroblasts in M6 AML may have focal acid phosphatase activity, which is localized to the Golgi zone [19]; they are usually positive for ANAE and ANBE [8]. These reactions differentiate M6 erythroblasts from the erythroblasts of congenital dyserythropoietic anaemia, in which acid phosphatase and NSE reactions are negative; however, positive reactions can also be seen in megaloblastic anaemia consequent on pernicious anaemia [8]. A Perls' stain for iron may show coarse siderotic granules; in a minority of cases numerous ring sideroblasts are present.

Table 1.10 Criteria for the diagnosis of acute myeloid leukaemia of M7 category (acute megakaryoblastic leukaemia).

- Blasts ≥30% of bone marrow nucleated cells
- Blasts demonstrated to be megakaryoblasts by immunological markers, ultrastructural examination or ultrastructural cytochemistry

Acute megakaryoblastic leukaemia: M7 acute myeloid leukaemia

Acute megakaryoblastic leukaemia was not included in the original FAB classification of AML but, following the demonstration that in some cases apparently undifferentiated blasts were actually megakaryoblasts, this category was added [3] (Table 1.10). M7 AML shows a markedly increased incidence in children with Down syndrome (see page 164). In infants and children M7 AML may be associated with t(1;22)(p13;q13) (see page 152), and in adults, and to a lesser extent in children, a significant proportion of cases are associated with abnormalities of chromosome 3q21q26 and with loss or long arm deletion of chromosome 5 or 7 [64]. Karyotypic abnormalities are more often found in M7 than in other FAB categories of AML (with the exception of M3) and the abnormalities are more often complex [64]. *GATA1* mutations are invariably present in Down syndrome-associated cases but are also sometimes seen in other cases [65]. Diagnosis is usually made by immunophenotyping, there being expression of platelet antigens such as CD41, CD42 and CD61. There is often coexpression of CD13, CD33 and CD7 [65]. Adult M7 AML, in comparison with other non-M3 AML, is associated with more adverse cytogenetic abnormalities, a lower complete remission rate and worse survival [66]. There is often an antecedent haematological abnormality and in one series of patients 19% were therapy related [66].

Leukaemic megakaryoblasts are often highly pleomorphic. Prominent and multiple nucleoli and cytoplasmic basophilia have been noted [19]. Binuclearity and clumping of blast cells have been noted to be frequent features [67]. In some cases the diagnosis can be suspected from the cytological features when the blasts show cytoplasmic protrusions or blebs, or when blasts coexist with apparently bare nuclei, with large bizarre platelets or with more mature cells showing megakaryocytic differentiation. Micromegakaryocytes are sometimes present and there may be emperipolesis [65]. In other cases the blasts cannot be distinguished from myeloblasts or resemble lymphoblasts, being small with a high nucleocytoplasmic ratio and with some chromatin condensation. The WBC is often reduced rather than elevated [68]. A minority of patients with M7 AML have thrombocytosis rather than thrombocytopenia. The percentage of bone marrow blasts may be underestimated as a result of fibrosis so that trephine biopsy can be important in assessment. The nature of megakaryoblasts may be suggested by the pattern of cytochemical reactions (see below) but a reliable identification requires immunophenotyping, ultrastructural examination (Fig. 1.43) or ultrastructural cytochemistry (Fig. 1.44). The clinicopathological picture designated acute myelofibrosis, i.e. pancytopenia with bone marrow fibrosis, usually represents acute megakaryoblastic leukaemia. There may be osteosclerosis as well as bone marrow fibrosis [69]. Other patients present with the usual features of AML, with hepatomegaly and splenomegaly being quite common. Cytological and histological features of M7 AML are shown in Figs 1.45 and 1.46. Some cases show some maturation to dysplastic megakaryocytes, as is shown in Fig. 1.47. With the exception of cases among children with Down syndrome, the prognosis in both children and adults appears to be poor [30,67,68].

Cytochemical reactions in M7 acute myeloid leukaemia

Megakaryoblasts are negative for MPO, SBB and CAE. The more mature cells of this lineage are PAS positive and have partially fluoride-sensitive NSE activity, demonstrated with ANAE. ANBE activity is demonstrable in only a minority of cases [67]. On PAS staining there are positive granules on a diffusely positive background. In some cases, those showing more cytoplasmic maturation, there are positive granules or block positivity, localized to the periphery of the cell or packed into the cytoplasmic blebs. A PAS stain can highlight the presence of micromegakaryocytes and megakaryoblasts with cytoplasmic maturation (Fig. 1.47). Esterase activity is usually multifocal punctate [67] but is sometimes localized to the Golgi zone [23,67]. There is a similar

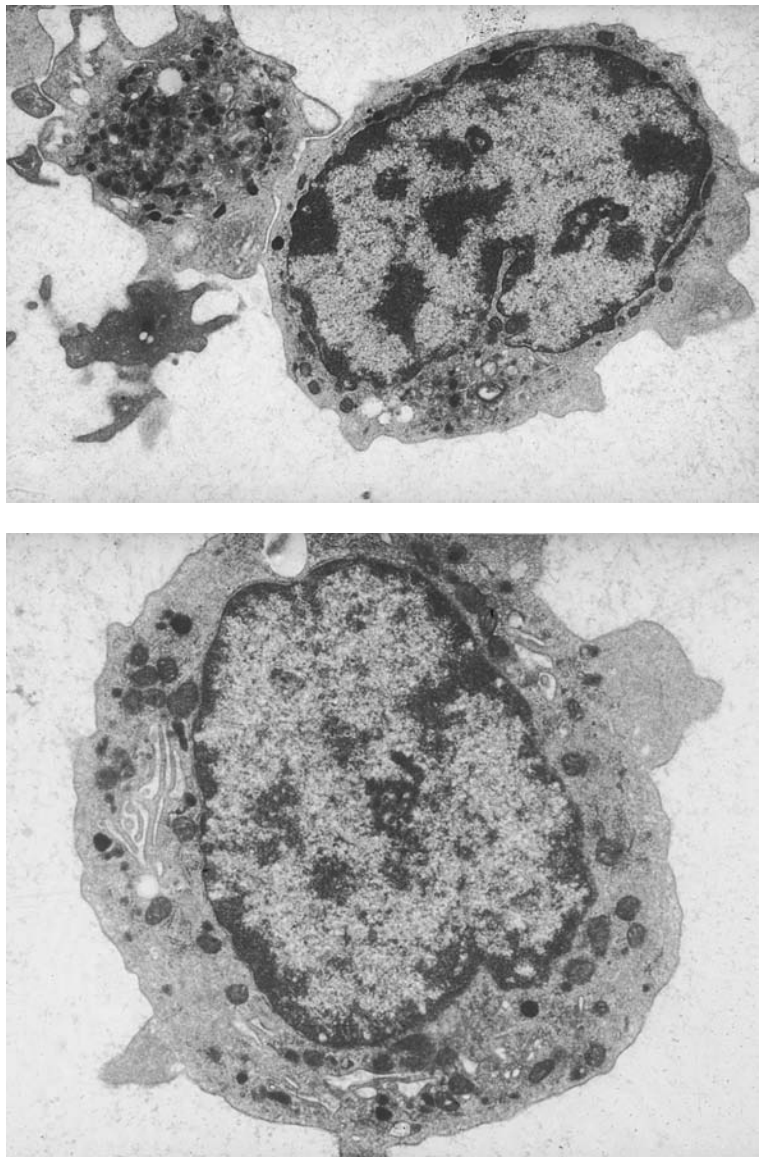


Fig. 1.43 Ultrastructural examination of peripheral blood cells from a patient with megakaryoblastic transformation of chronic granulocytic leukaemia showing: (a) a blast cell and a giant platelet; the megakaryoblast has characteristic granules including several bull's eye granules; (b) a megakaryoblast with platelet demarcation membranes. (By courtesy of Professor Daniel Catovsky.)

localization of acid phosphatase activity, which is tartrate sensitive [19]. In very immature megakaryoblasts, PAS and NSE reactions are negative.

Acute eosinophilic leukaemia

In the FAB classification, cases of eosinophilic leukaemia with a minimum of 30% bone marrow blast cells should be categorized as AML. They can be assigned to FAB categories with the addition of the abbreviation 'Eo' to indicate the eosinophilic differentiation, e.g. M2Eo and M4Eo. Such cases may have cardiac and other tissue damage as a consequence of release of eosinophil granule contents. Generally there is both neutrophilic and eosinophilic differentiation. Occasional cases show only eosinophilic differentiation. A minimum of 5% of bone marrow eosinophils has been suggested as a criterion for the recognition of significant

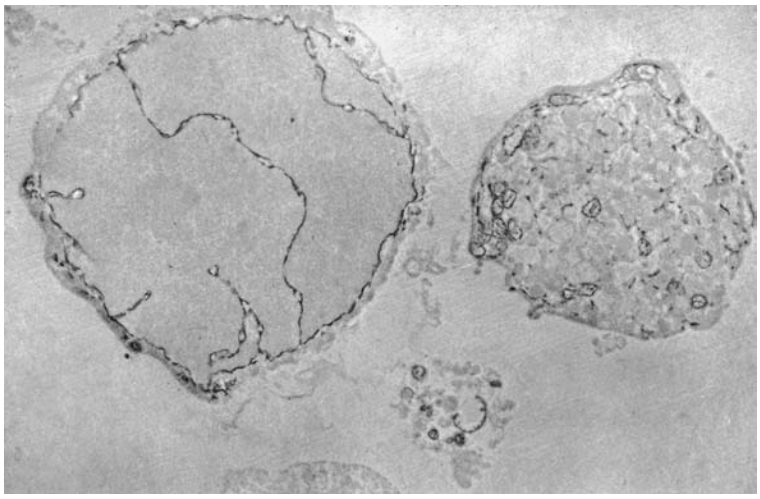
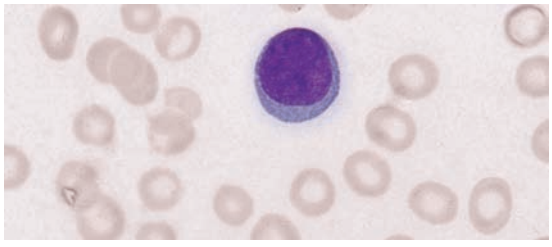
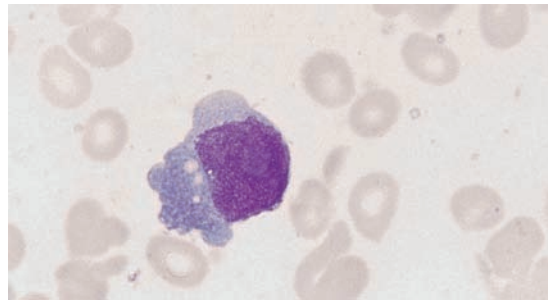


Fig. 1.44 Ultrastructural cytochemistry of a blast cell showing a positive platelet peroxidase reaction. (By courtesy of Professor Daniel Catovsky.)

(a)





(b)

Fig. 1.45 PB and BM films from a patient with FAB M7 AML presenting as acute myelofibrosis; the nature of the leukaemia was demonstrated by a positive reaction for platelet peroxidase. (a) PB film showing mild anisocytosis and a blast cell with no distinguishing features. (b) BM film showing a megakaryoblast. MGG ×100.

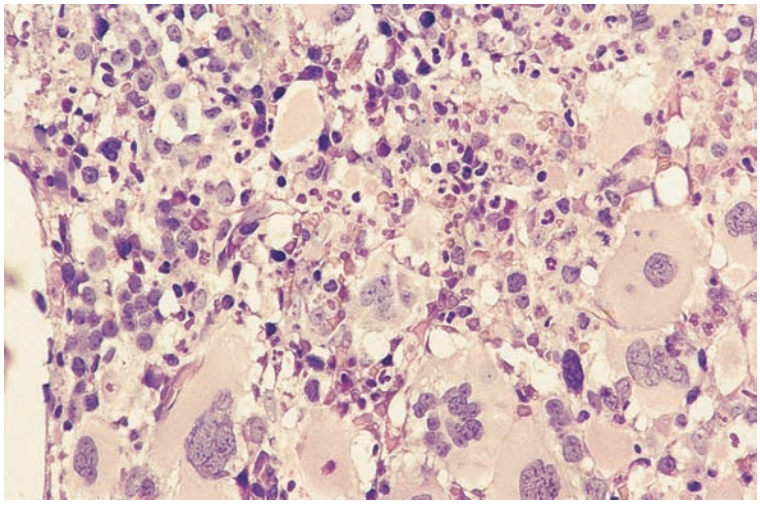


Fig. 1.46 Trephine biopsy section from a patient with FAB M7 AML showing increased blasts and large dysplastic megakaryocytes. Paraffin embedded, H&E ×40.

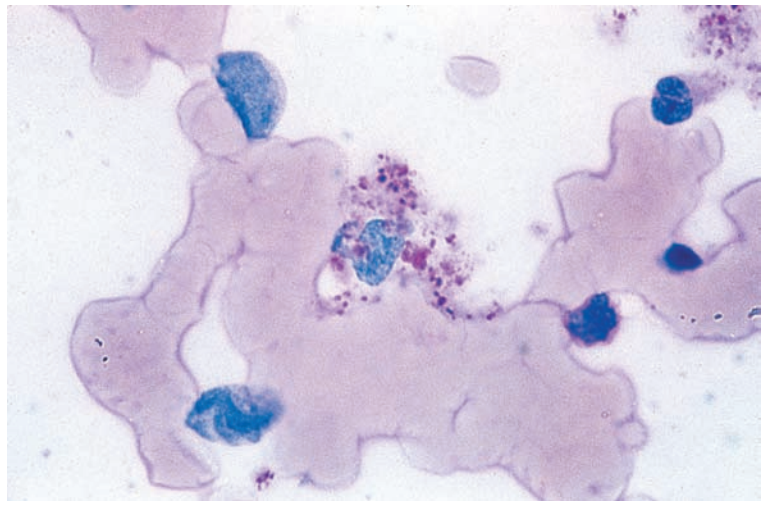


Fig. 1.47 BM film in FAB M7 AML showing a micromegakaryocyte with cytoplasmic blebs, which are PAS positive. PAS ×100.

eosinophilic differentiation [70]. In cases with maturation, eosinophils are readily recognizable by the characteristic staining of their granules. However, recognition of eosinophil precursors in M1Eo AML may require cytochemistry or the ultrastructural demonstration of characteristic granule structure (see Table 1.3), since primitive eosinophil granules differ little in their staining characteristics from the granules of neutrophil lineage myeloblasts (Fig. 1.48). Mature eosinophils often show vacuolation, degranulation and nuclear hyper- or hypolobulation. However, these cytological abnormalities are not specific for eosinophilic leukaemia, being seen also in reactive eosinophilia. The bone marrow in acute eosinophilic leukaemia sometimes shows the presence of Charcot–Leyden crystals, either free or within macrophages (Fig. 1.49). Occasionally similar crystals are seen within leukaemic cells (Fig. 1.50).

Cytochemistry in acute eosinophilic leukaemia Blast cells of eosinophil lineage are positive with MPO and SBB. With the SBB stain, the granule core may be left unstained. Peroxidase activity differs from that of the neutrophil lineage in being resistant to cyanide [51]. Cells of the eosinophil lineage are usually CAE negative but neoplastic eosinophils are

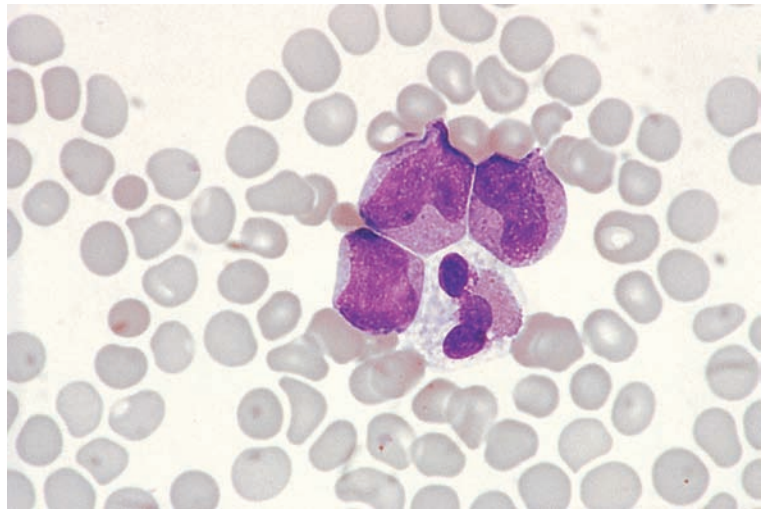


Fig. 1.48 PB film in acute eosinophilic leukaemia with abnormal eosinophil precursors showing a mixture of eosinophilic and azurophilic granules; maturing eosinophils are degranulated and some have nuclei of bizarre shapes. MGG ×100. (By courtesy of Dr Alistair Smith, Southampton.)

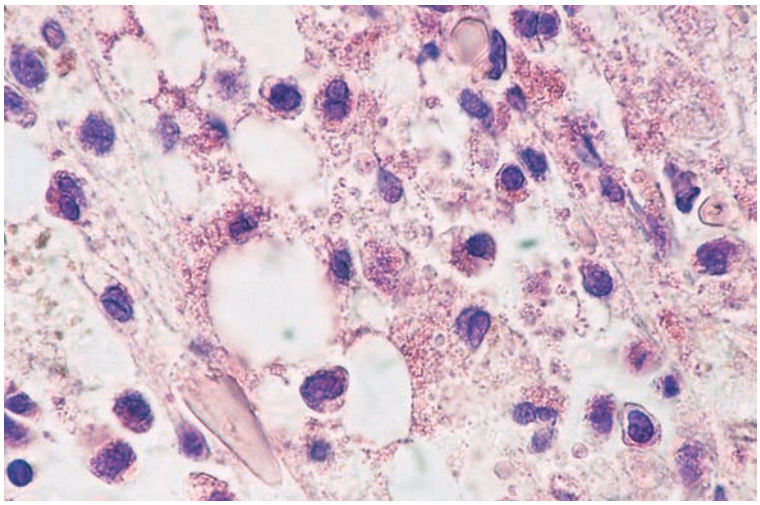


Fig. 1.49 Trephine biopsy section in acute eosinophilic leukaemia showing numerous eosinophils and part of a Charcot–Leyden crystal (same patient as Fig. 1.48). Paraffin embedded, H&E ×100. (By courtesy of Dr Alistair Smith and Dr Bridget Wilkins, London.)

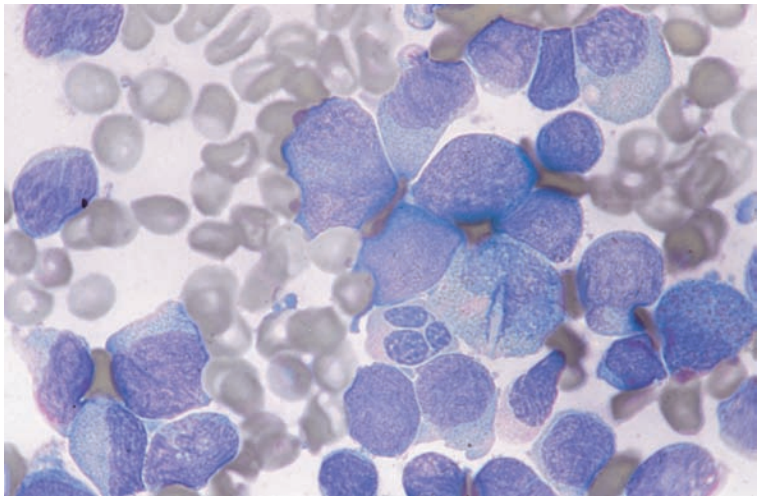


Fig. 1.50 BM film from a patient with acute eosinophilic leukaemia showing a Charcot–Leyden crystal within a leukaemic cell. MGG ×100.

sometimes positive (see page 137). A combined cytochemical stain for CAE and cyanide-resistant peroxidase activity is a convenient means of distinguishing cells of neutrophil and eosinophil lineage [51].

Acute basophilic leukaemia

In the FAB classification, cases of basophilic leukaemia with a minimum of 30% bone marrow blasts should be classified as AML. They can be assigned to FAB categories with the abbreviation 'Baso' to indicate the basophilic differentiation. Some cases show maturation and can be categorized as M2Baso or M4Baso. Others show very little maturation and fall into the M1Baso category. Cases that do not meet the minimal criteria for M1 AML but show evidence of basophil differentiation can be categorized as M0Baso. Cases of M2Baso and M4Baso AML usually have mixed neutrophilic and basophilic differentiation, whereas cases with very primitive basophil precursors (M1 and M0Baso AML) may show only basophilic differentiation. Cases of mixed phenotype acute leukaemia with basophilic, megakaryoblastic and T-lineage maturation have been recognized [71]. Patients with acute basophilic leukaemia do not usually show features

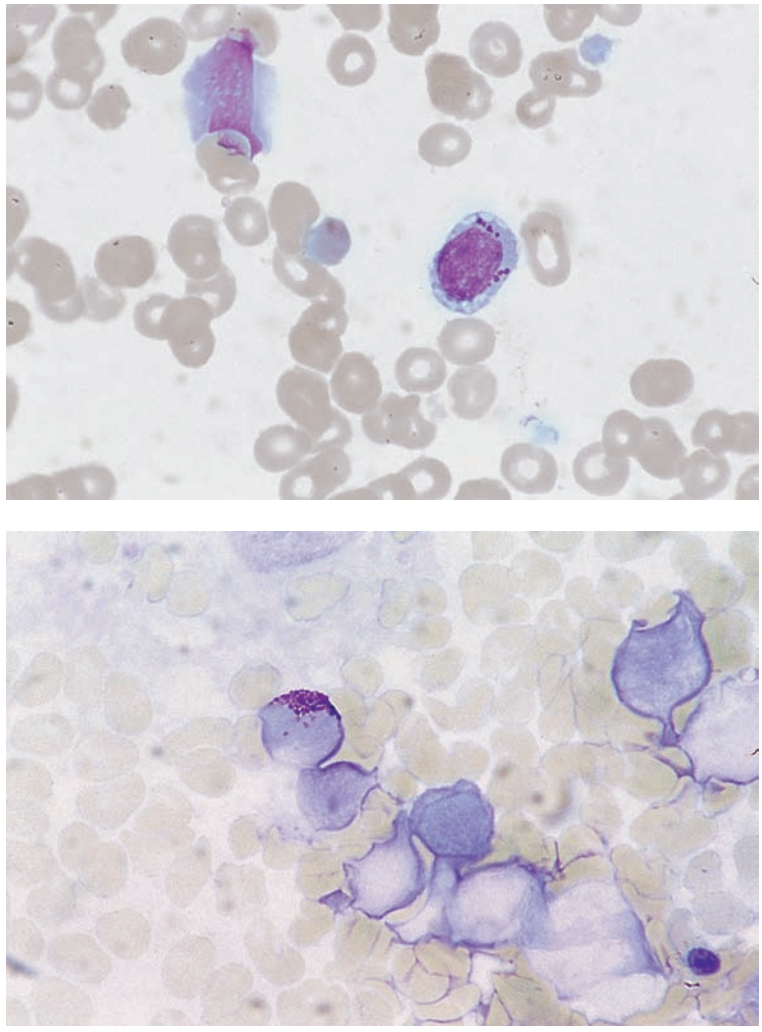


Fig. 1.51 BM film in acute basophilic leukaemia. (a) Vacuolated blast with large granules. MGG ×100. (b) Metachromatic staining with a toluidine blue stain. Toluidine blue ×100.

of histamine excess [72] but some patients have had urticaria, peptic ulceration or other gastrointestinal disturbance [73] and anaphylactoid reactions can occur following chemotherapy [74].

In cases with maturation, basophils are usually easily recognized by their cytological and cytochemical characteristics (Fig. 1.51). In other cases with little or no maturation, ultrastructural examination (see Table 1.3) is necessary. Sometimes there are granules with whorls or scrolls (characteristic of mast cells) in addition to typical basophil granules [74]. Blasts of basophil lineage may contain Auer rods [75].

Acute basophilic leukaemia is recognized in the WHO classification (see page 161).

Cytochemistry in acute basophilic leukaemia In acute basophilic leukaemia without maturation [76], SBB is commonly negative and MPO is negative by light microscopy. Often CAE is also negative, although it is weakly positive in later cells of basophil lineage. In cases showing maturation there is positivity with SBB, MPO and CAE, and metachromatic staining with toluidine blue, alcian blue and astra blue. Sometimes, staining with SBB is also metachromatic, granules being grey, black, pinkish or red

while granules of the eosinophil and neutrophil lineages are greenish-black. On MPO staining, blasts may have particularly coarse granules [71]. ε-amino caproate activity [77] is specific for the basophil lineage. At an ultrastructural level, ruthenium red can be used to identify basophil granules [76].

Acute mast cell leukaemia

Mast cell leukaemia can occur either *de novo* or as the terminal phase of systemic mastocytosis. It is not included in the FAB classification and in the WHO classification is regarded as a form of systemic mastocytosis. However, it should be noted that systemic mastocytosis terminates in other types of AML more often than in mast cell leukaemia. The peripheral blood shows mast cells (Fig. 1.52), which are often immature or morphologically abnormal with hypogranularity or nuclear lobulation. The bone marrow is hypercellular and infiltrated by mast cells (Fig. 1.53). Ultrastructural examination can confirm the diagnosis but it should be noted that in some cases cells show both basophil and mast cell characteristics [78]. As for acute basophilic leukaemia, anaphylactoid reactions may follow chemotherapy [79]. In some patients there is differentiation to both myeloblasts and mast blasts (Fig. 1.54) [80]. The term 'myelomastocytic leukaemia' has been suggested for these cases [81].

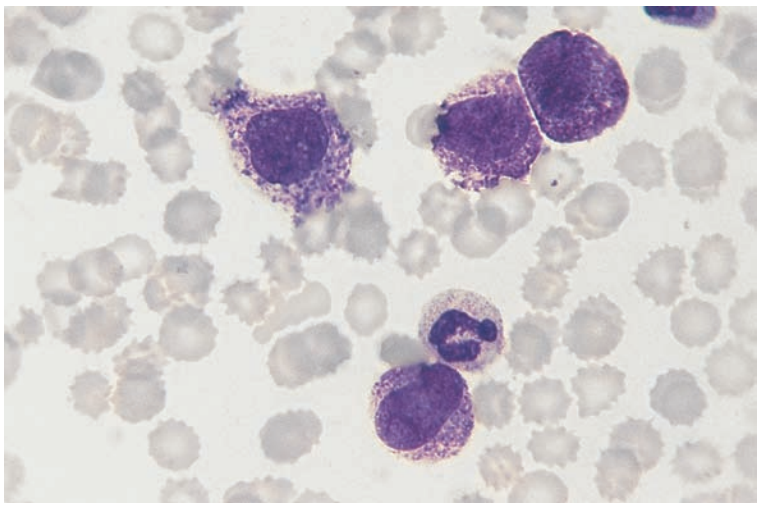


Fig. 1.52 PB film in mast cell leukaemia showing a neutrophil and four mast cells. MGG ×100. (By courtesy of Dr Ian Bunce and Miss Desley Scott, Brisbane.)

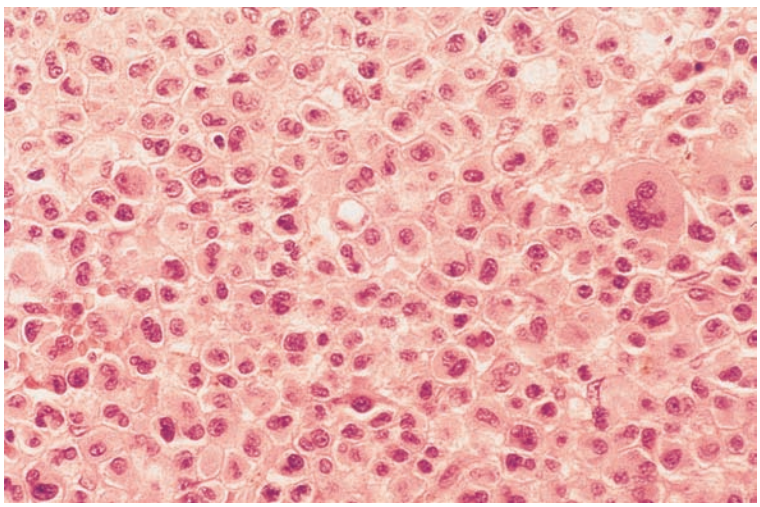
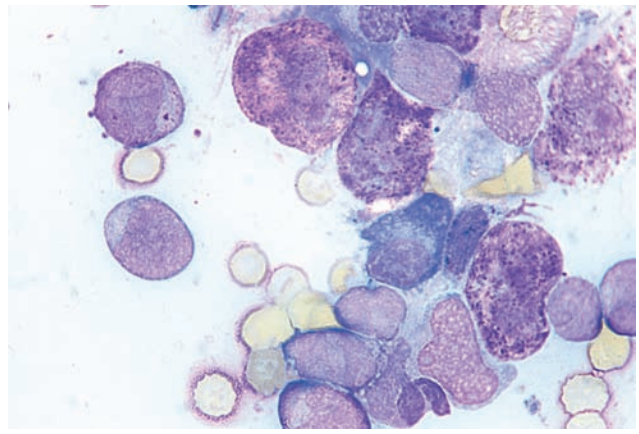


Fig. 1.53 Trephine biopsy section from a patient with acute mast cell leukaemia showing neoplastic mast cells with irregular nuclei and voluminous cytoplasm. Paraffin embedded, H&E ×40. (By courtesy of Professor Ghulam Mufti, London.)


(a)

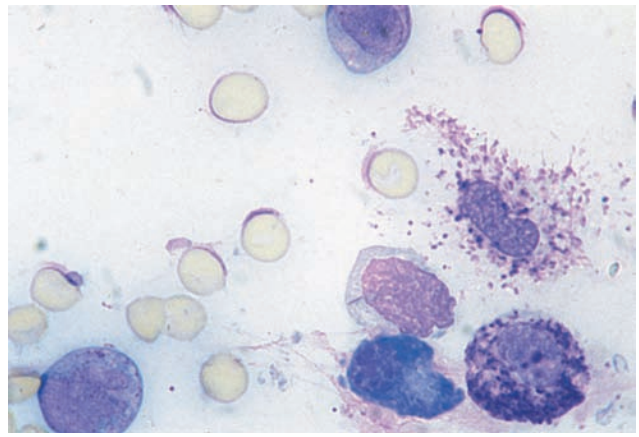

(b)

Fig. 1.54 BM film from a patient with acute leukaemia showing mast cell and neutrophilic differentiation, showing: (a) blast cells and immature abnormal mast cells; (b) abnormal mast cells and a blast cell containing an Auer rod. MGG ×100. (By courtesy of Dr Neelam Varma, Chandigar.)

Mast cell leukaemia can have an aleukaemic presentation [82]. Mutations in the *KIT* gene are found, including but not only the D816V mutation that is found in systemic mastocytosis; intragenic deletions have also been demonstrated [83].

Cytochemistry in acute mast cell leukaemia Mast cells stain metachromatically with a Giemsa stain and with toluidine blue, alcian blue and astra blue. They are CAE positive. There may be cytoplasmic crystals, which stain pink on a May–Grünwald–Giemsa (MGG) stain. When cells are relatively agranular, immunocytochemistry for mast cell tryptase is more sensitive than cytochemical staining (Fig. 1.55) [80]. They also express CD117.

Langerhans cell leukaemia

Rare cases of AML have leukaemic cells showing features of Langerhans cells [84] (Fig. 1.56). Such cases may occur *de novo* but it is likely that cases resembling M5 AML supervening in Langerhans cell histiocytosis [85] also represent a leukaemia of Langerhans cells. The diagnosis is made by assessment of cytology and immunophenotype (CD1a is expressed) with the demonstration of Birbeck granules by ultrastructural examination providing a definitive diagnosis.

Hypoplastic or hypocellular acute myeloid leukaemia

The majority of cases of AML have a hypercellular bone marrow. However, in a minority of cases the bone marrow is hypocellular. Hypoplastic AML has been variously defined as AML with bone marrow cellularity being less than 50% [86], less than 40% [87], less than 30% [88] or, in the WHO classification, less than 20% [89]. Hypoplastic AML can occur *de novo* or supervene in MDS. Often

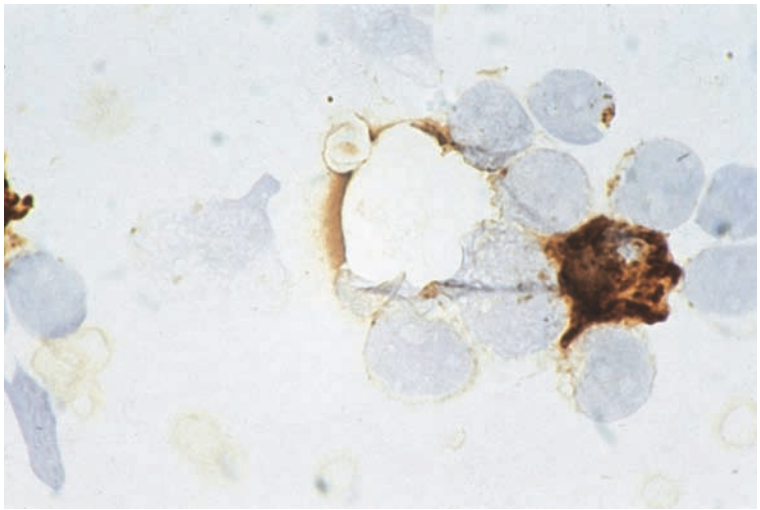


Fig. 1.55 BM film from a patient with acute mast cell leukaemia showing a mature mast cell packed with granules that are strongly positive for mast cell tryptase and several blast cells with tryptase-positive granules; these latter cells are therefore identified as blast cells of mast cell lineage (same patient as Fig. 1.54). Immunoperoxidase ×100. (By courtesy of Dr Neelam Varma and Dr Bridget Wilkins.)

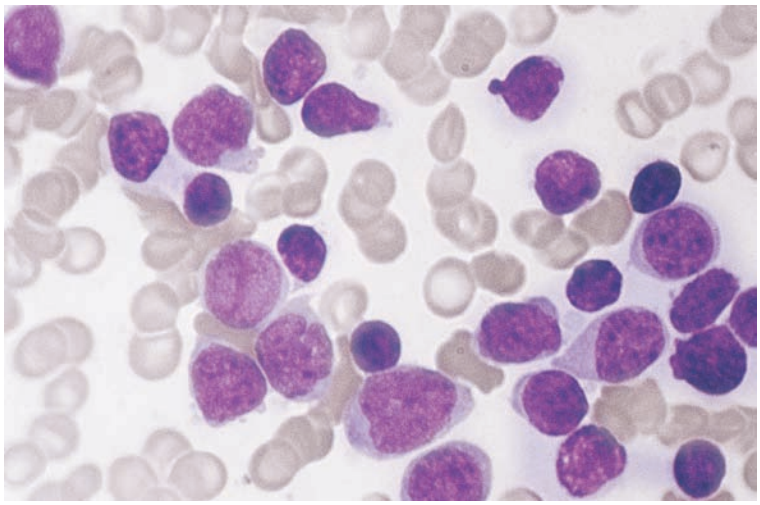


Fig. 1.56 Langerhans' cell leukaemia. MGG ×100. (By courtesy of Dr B. I. S. Srivastava, Buffalo, NY.)

examination of the peripheral blood and bone marrow does not permit a distinction from MDS since there is often pancytopenia with few circulating blast cells and a hypocellular bone marrow aspirate. Diagnosis is then dependent on identifying more than 30%* of blast cells on examination of bone marrow trephine biopsy sections. Hypocellular AML can be assigned to FAB categories, often falling into M0, M1 or M2 categories. Because of the high percentage of lymphoid cells in hypocellular AML it has been suggested that the FAB criteria for the diagnosis of AML should be modified in respect to this subtype so that blasts are counted as a percentage of all nucleated cells with the exception of lymphocytes [90]. Hypocellular AML often has a smouldering clinical course. However, intensive chemotherapy often achieves a complete remission, which may be associated with restoration of normal bone marrow cellularity [90].

* It should be noted that the criterion of at least 30% blast cells has been altered, in the WHO classification, to at least 20% blast cells (see page 115).

Clinical correlates of FAB categories of acute myeloid leukaemia

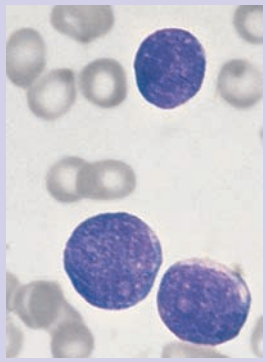
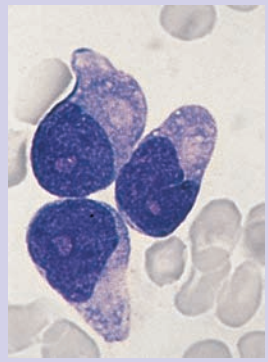
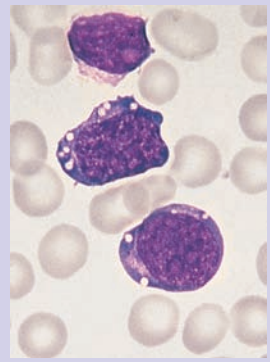
The FAB category of M3 AML is a distinct disease entity (see page 125). Otherwise there are only minor clinical differences between FAB categories. M4 and M5 AML are associated with more hepatosplenomegaly, skin infiltration and gum infiltration. M0 AML [91] and M6 AML are associated with complex cytogenetic abnormalities and with a worse prognosis than other categories.

The FAB classification of acute lymphoblastic leukaemia

Initially, acute lymphoblastic leukaemia was largely a diagnosis of exclusion. Although some cases had characteristic cytological features, others were categorized as 'lymphoid' only because they did not show any definite cytological or cytochemical evidence of myeloid differentiation. With the availability of a wide range of monoclonal antibodies directed at antigens expressed on lymphoid cells, the diagnosis of ALL is now based on positive criteria. The role of immunophenotyping in the diagnosis and classification of ALL will be discussed in detail in Chapter 2. It is sufficient at this stage to say that ALL is classified broadly as B lineage and T lineage. B-lineage ALL includes a small minority of cases with the immunophenotypic features of mature B cells (regarded as non-Hodgkin lymphoma rather than as ALL in the WHO classification) and a large majority of cases with the immunophenotype of B-cell precursors. The latter group includes a major subset designated common ALL, expressing a surface membrane antigen known as the common ALL antigen or CD10.

The FAB group have assigned ALL to three cytological categories: L1, L2 and L3. The classification is summarized in Table 1.11. Apart from a strong correlation between L3 cytological features and a mature B phenotype there is little relationship between the cytological features and the

Table 1.11 Morphological features of acute lymphoblastic leukaema subtypes.

FAB category	L1 ALL	L2 ALL	L3 ALL
			
Cell size	Mainly small	Large, heterogeneous	Medium to large, homogeneous
Nuclear chromatin	Fairly homogeneous, may be condensed in some cells	Heterogeneous	Finely stippled, homogeneous
Nuclear shape	Mainly regular	Irregular; clefting and indentation common	Regular; oval or round
Nucleolus	Not visible or small and inconspicuous	Usually visible, often large	Usually prominent
Amount of cytoplasm	Scanty	Variable, often abundant	Moderately abundant
Cytoplasmic basophilia	Slight to moderate	Variable	Strong
Cytoplasmic vacuolation	Variable	Variable	Often prominent

immunophenotype. The recognition of L3 ALL is generally straightforward but the categorization of a case as L1 or L2 can be difficult. However, it is of little clinical significance whether the cytological features are those of L1 or L2 ALL.

It should be noted that although myeloblasts do not show any appreciable chromatin condensation, lymphoblasts may do so. This is often noticeable in some of the smaller blasts in common ALL of L1 type. It has also been noted that a minority of cases of T-lineage ALL, particularly those with a relatively mature immunophenotype, have leukaemic cells that are difficult to recognize as blasts because of chromatin condensation and inconspicuous nucleoli [92]; immunophenotyping is of importance in these cases.

Acute lymphoblastic leukaemia of L1 subtype

In L1 ALL [1] small cells, up to twice the diameter of a red cell, predominate. They have a high nucleocytoplasmic ratio. The nucleus is regular in shape with only occasional clefting or indentation, the chromatin pattern is fairly homogeneous (although smaller cells may show a greater degree of chromatin condensation) and the nucleoli, if visible at all, are small and inconspicuous. The scanty cytoplasm is slightly to moderately basophilic, rarely intensely basophilic, and in some cases shows a variable degree of vacuolation. In a minority of cases there are small numbers of azurophilic granules. Typical examples of L1 ALL are shown in Figs 1.57 and 1.58 and ultrastructural features in Fig. 1.59. The L1 category includes the majority of cases of ALL; in childhood 70–80% of cases fall into this category. L1 ALL may be of B or T lineage.

Acute lymphoblastic leukaemia of L2 subtype

In L2 ALL [5] the blasts are larger and more heterogeneous. The nucleocytoplasmic ratio is variable from cell to cell but the cytoplasm, which shows a variable degree of basophilia, may be moderately

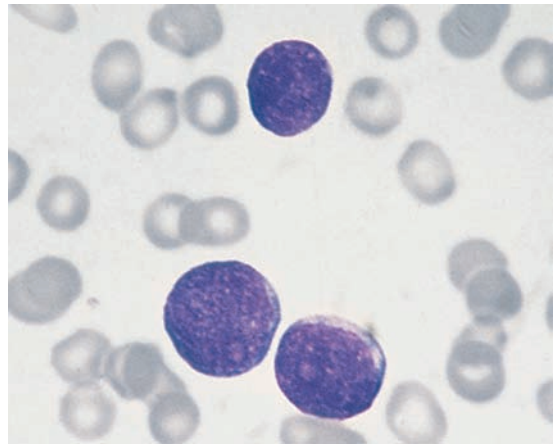


Fig. 1.57 PB film of a patient with FAB L1 acute lymphoblastic leukaemia (ALL). MGG ×100.

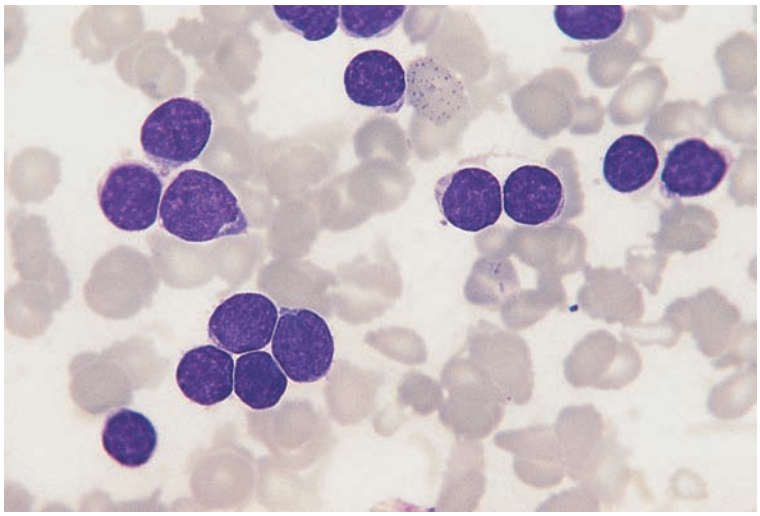


Fig. 1.58 BM film from a patient with FAB L1 ALL. MGG ×100.

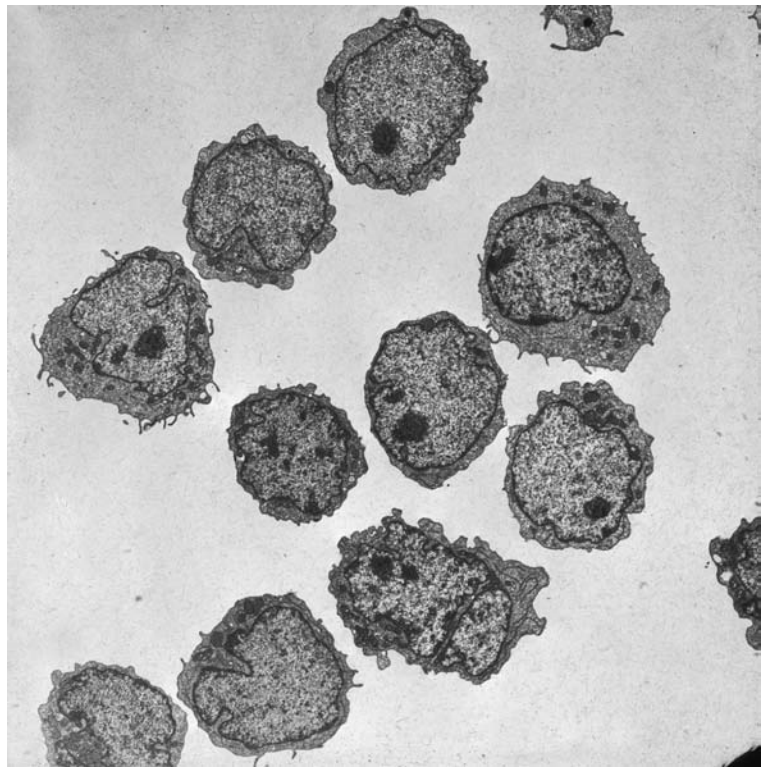


Fig. 1.59 Ultrastructure of lymphoblasts in FAB L1 ALL. (By courtesy of Professor Daniel Catovsky.)

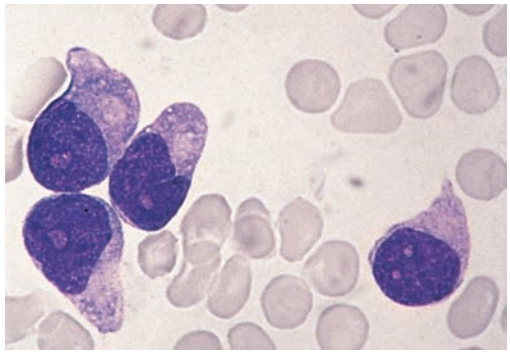


Fig. 1.60 BM film from a patient with FAB L2 ALL showing large pleomorphic blasts; the cells were CD10 positive. MGG ×100.

abundant. The nuclei are irregular in shape with clefting, folding and indentation being common, and with heterogeneity also of the chromatin pattern. Nucleoli are usually present and may be large. A variable degree of cytoplasmic vacuolation may be present, and in a minority of cases there are small numbers of azurophilic, but peroxidase-negative, granules. Typical examples of L2 ALL are shown in Figs 1.60–1.62. About a quarter of cases of ALL fall into the L2 category. L2 ALL may be of B or T lineage.

Acute lymphoblastic leukaemia of L3 subtype

In L3 ALL [5] the blast cells are medium to large and homogeneous. The nucleocytoplasmic ratio is lower than in L1 ALL. The nucleus is regular in shape, varying from round to somewhat oval. The chromatin pattern is uniformly stippled or homogeneous, with one or more prominent, sometimes vesicular, nucleoli. In contrast to L1 and L2 ALL, in which mitotic figures are uncommon, the mitotic index is high and many apoptotic cells are seen. The cytoplasm is strongly basophilic with variable but prominent vacuolation. Typical examples of L3 ALL are shown in Figs 1.63–1.66. L3 ALL constitutes only 1–2% of cases of ALL.

L3 ALL is often the leukaemic equivalent of Burkitt lymphoma. An aspirate from a lymph node or other tissue affected by this lymphoma shows

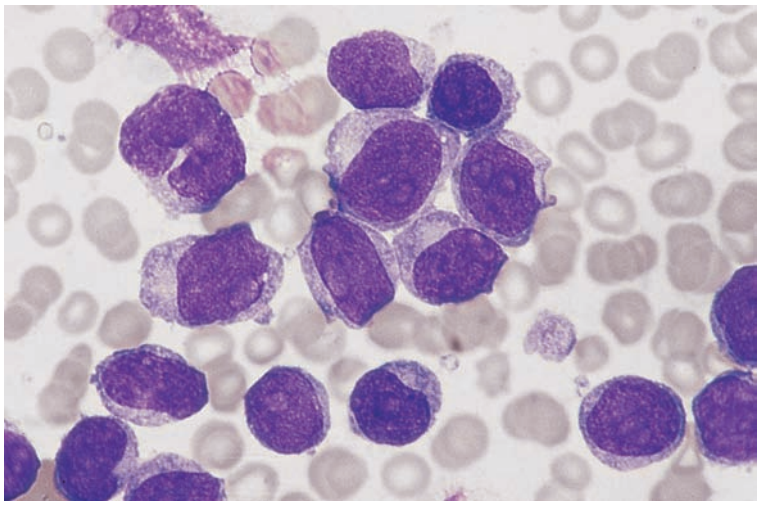


Fig. 1.61 BM film from a patient with FAB L2 ALL showing medium to large pleomorphic blasts which were CD10 negative but positive for CD19, HLA-DR and TdT. MGG ×100.

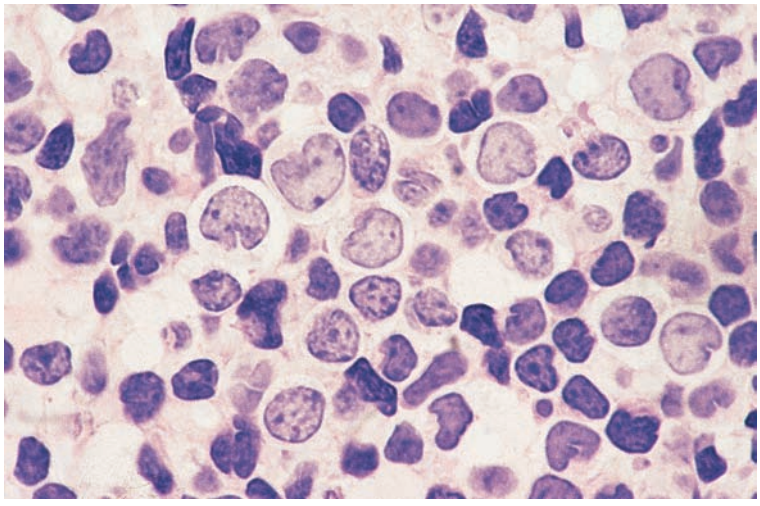


Fig. 1.62 Trephine biopsy section from a patient with FAB L2 ALL. Resin embedded, H&E ×100.

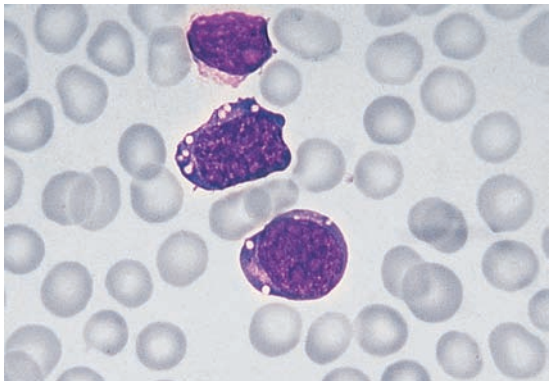


Fig. 1.63 PB film of a patient with FAB L3 ALL with the immunological phenotype being mature B cell. MGG ×100.

cells that are cytologically, immunophenotypically and cytogenetically the same as those of L3 ALL. Such cases are no longer classified as ALL.

The great majority of cases with the cytological features of L3 ALL have a mature B-cell immunophenotype, i.e. they express surface membrane immunoglobulin (SmIg). Less often cases have a common ALL phenotype, a pre-B immunophenotype (cytoplasmic immunoglobulin positive) [93] or even a T-cell [94] (Fig. 1.65) or a hybrid B–T phenotype [95]. Cases have also been reported of acute leukaemia with L3 morphology with a lack of B or T markers but with the characteristics of very early erythroid cells [96,97]; as these latter cases had cytogenetic findings usually associated with L3 ALL

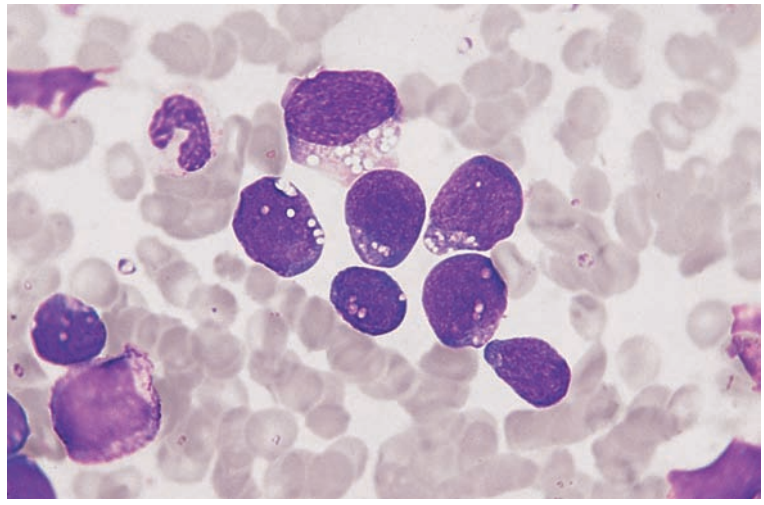


Fig. 1.64 BM film of a patient with FAB L3 ALL with the immunological phenotype being mature B cell. MGG ×100.

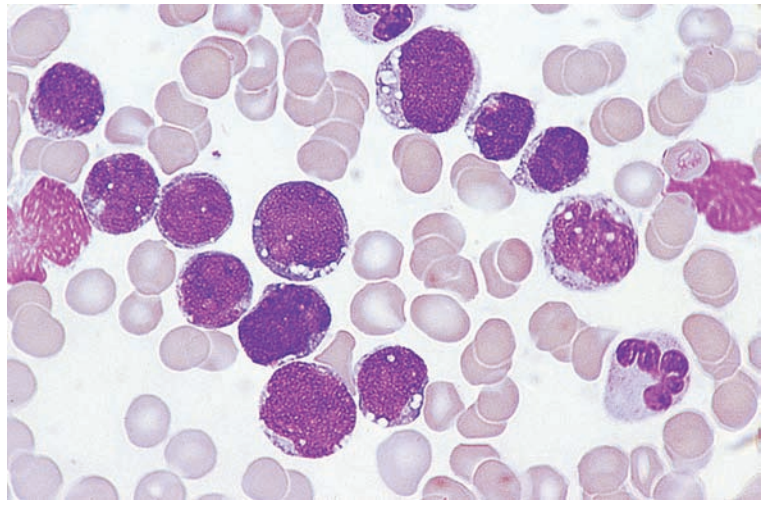


Fig. 1.65 PB film a case of FAB L3 ALL which was unusual in being of T lineage and having a t(7;9) translocation. MGG ×100.

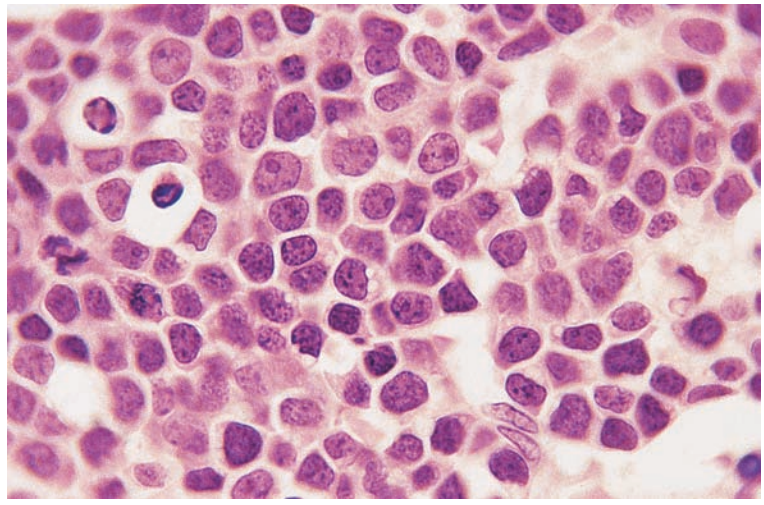


Fig. 1.66 Trephine biopsy section from a patient with FAB L3 ALL, B-cell phenotype; vacuolation of some of the blasts can be observed and there are two blasts undergoing apoptosis. Paraffin embedded, H&E ×100.

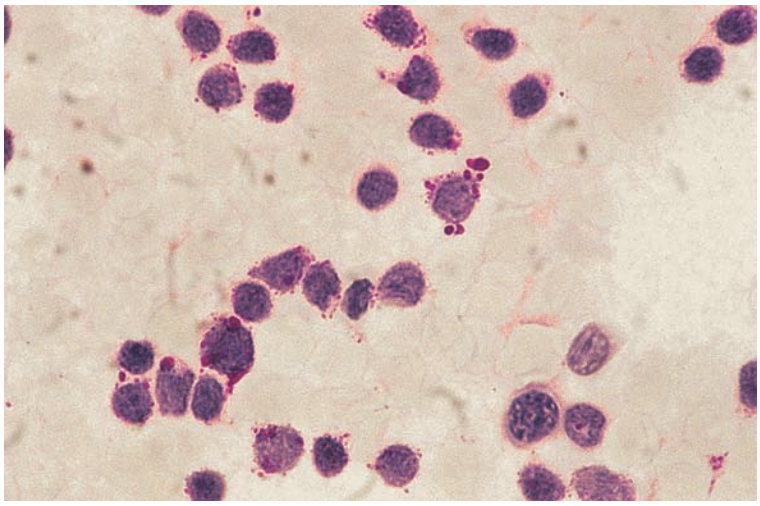


Fig. 1.67 PAS stain of the BM of a patient with common ALL showing block positivity. PAS ×100. (By courtesy of Dr Ayed Eden, Southend-on-Sea.)

or Burkitt lymphoma (see page 338) the involvement of a primitive cell with the potential for both B lymphoid and erythroid differentiation is suggested. Rarely L3 morphology can be found in association with mixed lineage acute leukaemia [98], acute myelomonocytic leukaemia, small cell carcinoma of the lung [99] or undifferentiated carcinoma [100].

When a patient shows L3 cytological features, further investigation is essential and is urgent. This should be initially immunophenotyping and, in B-lineage cases, cytogenetic or molecular genetic analysis. Cases with a mature B immunophenotype may have specific Burkitt lymphoma-related translocations; they do poorly with standard ALL management but have a much more favourable prognosis with specific protocols. Patients with L3 morphology and a mature B immunophenotype may also have t(14;18)(q32;q21); their prognosis is poor and optimal management has not been defined. Rare cases with L3 cytological features have had t(9;22)(q34;q11.2) (see page 180). A B-cell precursor immunophenotype and t(1;19)(q23;p13) may also be found (see page 186); they do not have an adverse prognosis and should be treated as ALL, not with protocols relevant to Burkitt lymphoma.

Only when FAB L3 cytological features are associated with a precursor-B (or precursor-T) immunophenotype, can the diagnosis of ALL be sustained. Other cases are usually non-Hodgkin lymphoma, particularly Burkitt lymphoma.

Cytochemistry of acute lymphoblastic leukaemia There is little relationship between cytochemical reactions and the FAB categories, but somewhat more between cytochemical reactions and immunophenotypic categories.

Lymphoblasts show negative reactions for MPO and CAE. With SBB, very fine, positive cytoplasmic granules may be present but these are usually obscured by the counterstain so that for practical purposes SBB is negative [8]; these very fine granules probably represent mitochondria. Rare cases of apparent ALL have shown coarse granular positivity with SBB [8,101]. In ALL the great majority of neutrophils are MPO positive and show strong positivity with SBB, whereas in AML there may be an expanded population of SBB- and MPO-negative neutrophils.

In B-lineage ALL, the PAS stain often shows characteristic block positivity (Fig. 1.67); this is seen also, although perhaps less often, in T-lineage ALL. The blocks and coarse granules of positively staining material are present in PAS-negative cytoplasm, whereas in the case of the block positivity that is seen much less often in cases of AML (mainly in monoblasts and erythroblasts) the PAS-positive blocks are in cells with a background of diffuse or finely granular positivity (see Fig. 1.35e). L3 ALL is usually PAS negative [102].

There is some correlation between PAS positivity and blast vacuolation. In one study of 733 children [103], 28% had more than 10% of vacuolated

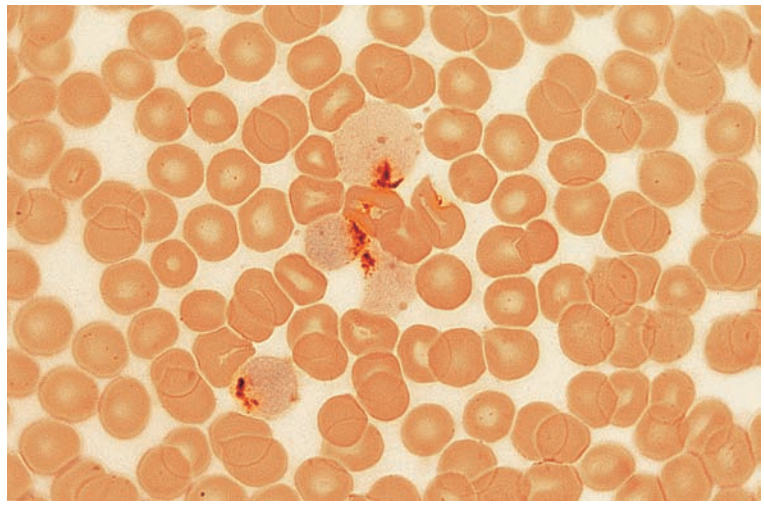


Fig. 1.68 Acid phosphatase stain of the PB of a patient with T-lineage ALL showing focal positivity. Acid phosphatase reaction ×100.

blasts. This finding correlated strongly with PAS positivity, a relatively low WBC and CD10 expression. When cases had both vacuoles and PAS positivity, the chance of CD10 being positive was 98%. Although PAS staining can be useful in the diagnosis of ALL it is important to recognize that PAS block positivity alone is not a sufficient basis for this diagnosis.

The presence of strong localized positivity for acid phosphatase is common in T-lineage ALL (Fig. 1.68) but rare in B-lineage ALL. This pattern should not, however, be regarded as pathognomonic for T-lineage ALL as a similar pattern of staining is not uncommon in M6 AML and may also be seen in M7 AML [10]. In a minority of cases of ALL, the presence of azurophilic granules on the Romanowsky stain can be related to the presence of lysosomal granules, which also show punctate acid phosphatase activity [104]. This phenomenon correlates with B-lineage ALL (mainly common ALL) and with L2 morphology. T lymphoblasts may also have localized coarse granular positivity for NSE (NASDA, ANAE and ANBE), whereas B-lineage blasts either give negative reactions or have scattered fine granules. Neither pattern resembles the strong generalized positivity that is characteristic of cells of monocyte lineage.

In L3 ALL, the vacuoles stain with oil red O, demonstrating that they contain lipid [102]. However, oil red O-positive vacuoles are sometimes seen in L1 and L2 ALL, and were also noted in the case of metastatic carcinoma that simulated L3 ALL [100].

Because of their lack of specificity, cytochemical stains should be regarded as redundant in the diagnosis of ALL unless immunophenotyping is unavailable [105] and, when used, there must be a constant awareness of their lack of specificity.

Clinical correlates of FAB categories of acute lymphoblastic leukaemia

Many cases of L3 'ALL' represent Burkitt lymphoma and the cytological features are important in suggesting this diagnosis. However, the categorization of a case as L1 or L2 ALL is of little importance. The FAB L1 category includes more childhood cases with a relatively good prognosis. The incidence of L1 ALL falls with increasing age whereas the incidence of L2 ALL does not vary much with age. L2 ALL has generally been found to have a worse prognosis, although the difference is not major. In some series the prognostic difference disappeared if age was allowed for, whereas in other series the FAB category was an independent prognostic factor [106]. The immunophenotype and, to an even greater extent, the cytogenetic and molecular genetic characteristics are of much greater relevance to prognosis and treatment choice than is the FAB category.

Problems with the FAB classification of acute leukaemia

The FAB classification has been very widely used but since its inception there have been criticisms.

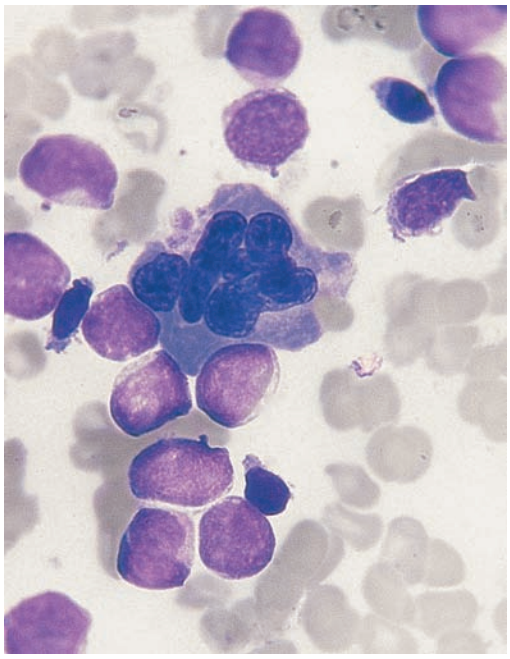


Fig. 1.69 BM film of a patient with FAB M1 AML showing a giant erythroid cell. MGG ×100.

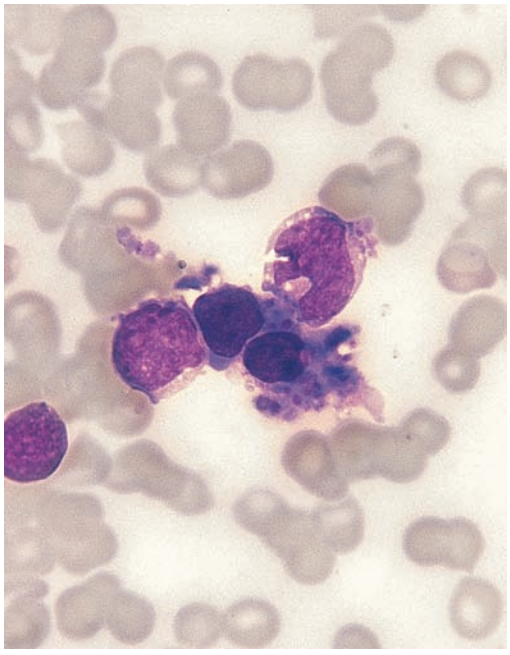


Fig. 1.70 BM film of a patient with FAB M4 AML showing two micromegakaryocytes. MGG ×100.

These centred on: (i) the necessarily arbitrary criteria for FAB categories; (ii) the lack of reproducibility between observers; (iii) the appropriateness of the criteria for distinguishing between AML and MDS, particularly the requirement for 30% of bone marrow blast cells; (iv) the occurrence of unclassifiable cases; and (v) the failure to include all relevant information in the classification, e.g. there is no consideration of the presence of bi- or trilineage dysplasia (Figs 1.69 and 1.70) or of cytogenetic and molecular genetic abnormalities, and immunophenotyping had only a limited role so that mixed phenotype acute leukaemia was not recognized. These criticisms have been largely, although not entirely, addressed by later classifications incorporating new methods of investigation (see Chapter 3) and do not detract from the major significance of the FAB classifications. These classifications brought clarity to a previously confused area. The degree of standardization that was achieved within and between countries greatly improved communication between haematologists and provided a framework for the major advances that subsequently occurred in the genetics and molecular genetics of haematological neoplasms. No other proposed classification was so widely accepted nor stood the test of time. However, after several decades of dominance, the FAB classification is now being supplemented or replaced by the WHO classification.

Results of automated full blood counts in acute leukaemia

Modern automated instruments that perform full blood counts detect the majority of cases of AML and ALL, identifying blast cells by means of their light-scattering, cytochemical and other characteristics. The current automated Siemens Advia 120 instrument includes peroxidase cytochemistry and produces scatterplots similar to those of earlier Bayer instruments, which are of some use in the further classification of AML [107] (Fig. 1.71). Cases of ALL and FAB M0 and M7 AML show an abnormal cluster of large, peroxidase-negative cells (Fig. 1.71b). In M1 AML it is apparent that blasts have peroxidase activity (Fig. 1.71c) and in M2 AML the peroxidase activity is stronger (Fig. 1.71d), giving a higher mean peroxidase score. In M3 and

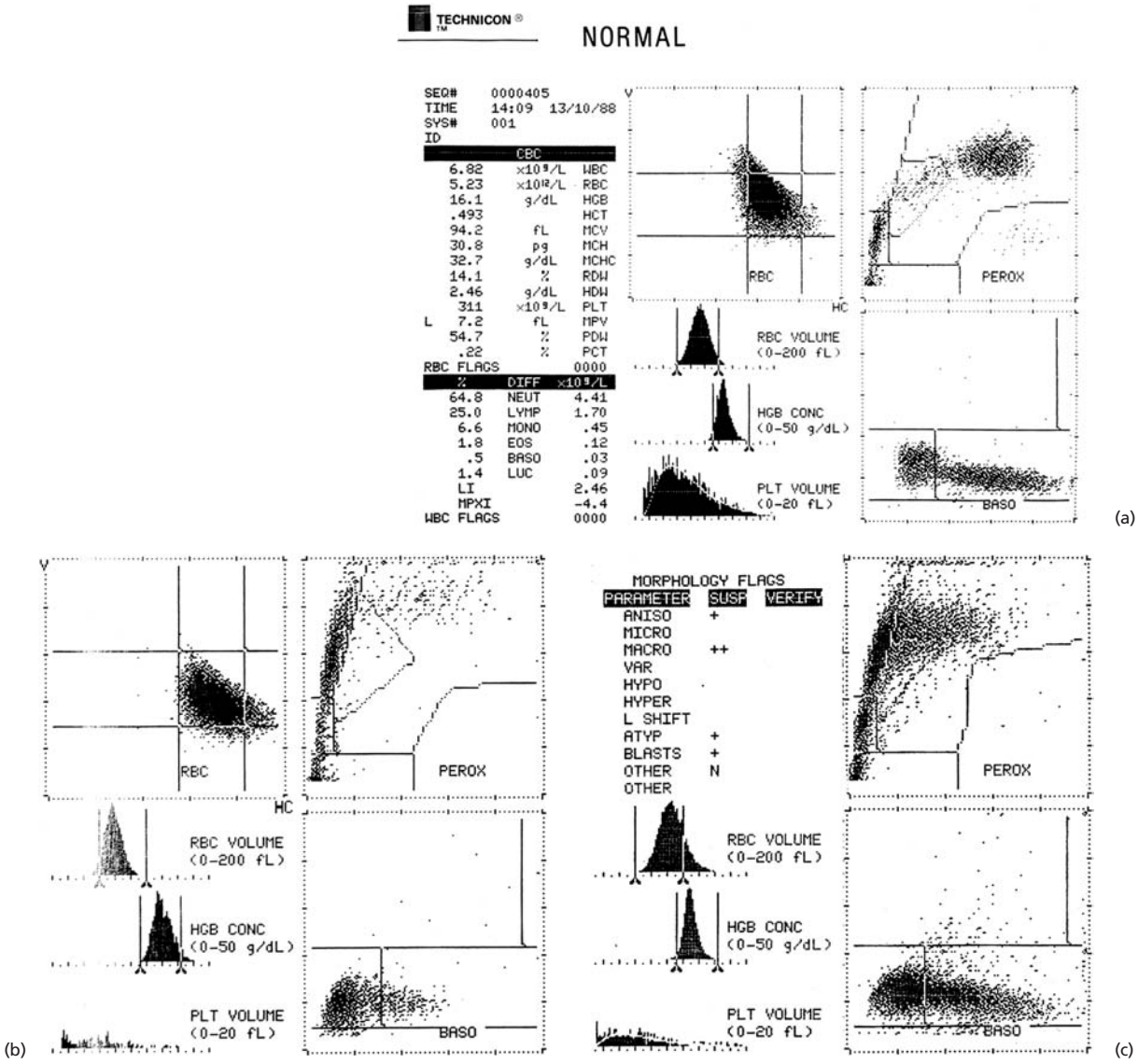


Fig. 1.71 Printouts from Bayer-Technicon H.1 series instruments on blood samples from a healthy volunteer and from patients with AML. (a) Histograms, red cell cytogram and scatterplots on a normal blood sample using a Bayer-Technicon H2 automated blood cell analyser. In the peroxidase cytogram separate clusters are identified, which represent neutrophils, eosinophils, monocytes, lymphocytes and 'large unstained (i.e. peroxidase-negative) cells' (LUC); in the basophil-lobularity channel there is a rounded head, which represents mononuclear cells (monocytes and lymphocytes) and an extended tail, which represents neutrophils and eosinophils. Basophils fall above the horizontal threshold. (b) Histograms, red cell cytogram and scatterplots on a blood sample from a patient with FAB M0 AML performed on a Bayer-Technicon H2 automated analyser. The blasts are peroxidase negative and therefore fall into the LUC area; the only indication that this is AML not ALL is that the neutrophil cluster is more dispersed than normal indicating neutrophil dysplasia. Note also the dense mononuclear cluster expanded leftwards in the basophil-lobularity channel, which indicates the presence of blast cells. The platelet histogram shows that there is severe thrombocytopenia. Similar scattergrams to this are also seen in FAB M7 AML. (c) Histograms and scatterplots on a blood sample from a patient with FAB M1 AML performed on a Bayer-Technicon H2 automated analyser. Some of the blasts fall into the LUC area but others have peroxidase activity and thus fall into the areas normally occupied by monocytes and neutrophils; the platelet histogram shows thrombocytopenia. (*Continued*)

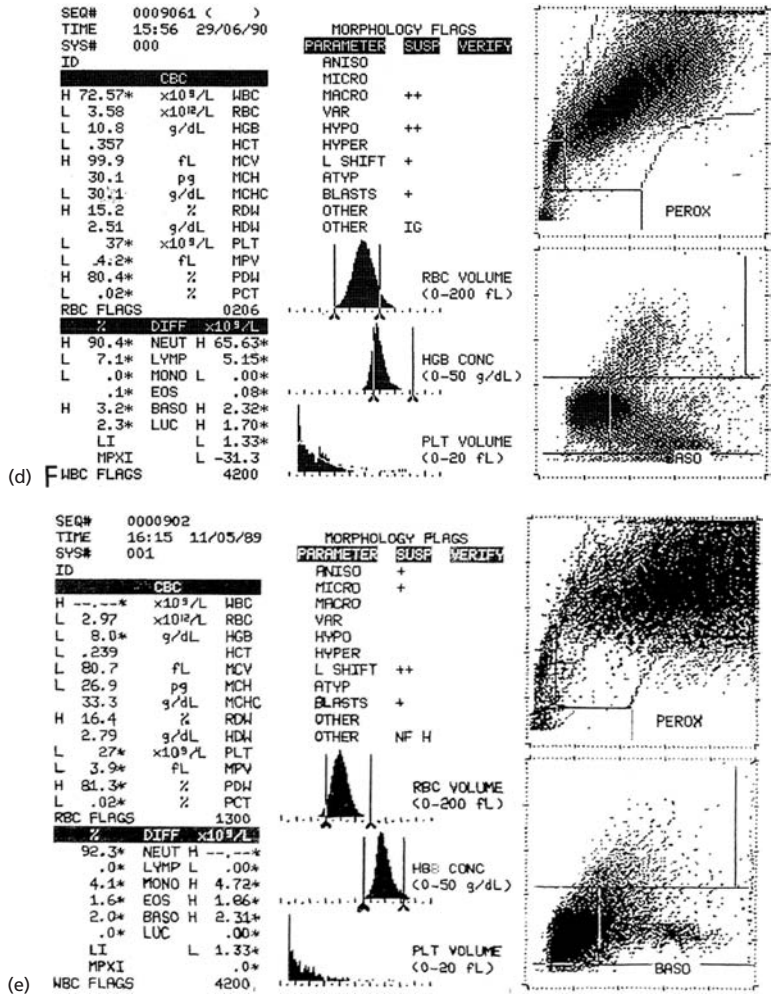
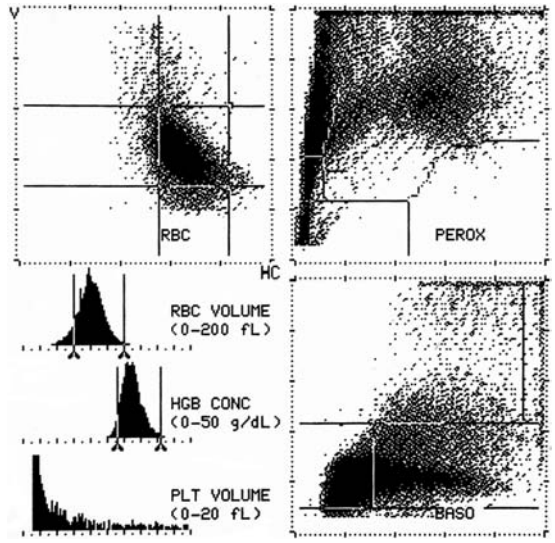


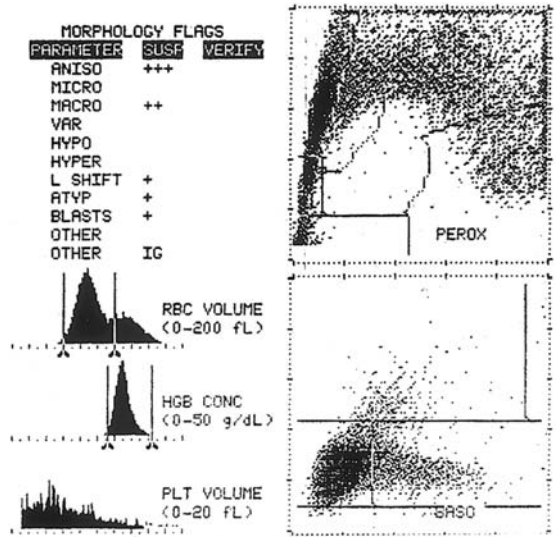
Fig. 1.71 (*Continued*) (d) Histograms and scatterplots on a blood sample from a patient with FAB M2 AML performed on a Bayer-Technicon H2 automated analyser. The blasts show more peroxidase activity than those in the case of FAB M1 AML, falling further to the right in the peroxidase histogram. The basophil-lobularity histogram shows the presence of blast cells expanding the mononuclear cluster leftwards and, in addition, causing pseudobasophilia since some of them fall in the area normally occupied by basophils; there is also thrombocytopenia. (e) Histograms and scatterplots on a blood sample from a patient with FAB M3V AML performed on a Bayer-Technicon H2 automated analyser. The abnormal promyelocytes are intensely peroxidase positive and form a triangular cluster based on the right-hand margin; there is pseudobasophilia and thrombocytopenia. The scattergrams in FAB M3 AML show the same features as are shown in this case of FAB M3V AML.

M3V AML there is very strong peroxidase activity, giving a characteristic scatterplot, which can provide rapid confirmation of a provisional diagnosis of M3V AML (Fig. 1.71e). M4 (Fig. 1.71f, g) and M5 AML show blasts cells with variable peroxidase activity.

ABX and related instruments employ Sudan black B instead of peroxidase cytochemistry and give similar information to Siemens/Bayer instruments. Other automated instruments, e.g. those produced by Beckman-Coulter, Sysmex and Abbott, also produce abnormal scatterplots in acute leukaemia. These show some difference between AML and ALL but do not differentiate well between FAB subclasses [108].



(f)



(g)

Fig. 1.71 (*Continued*) (f) Histograms, red cell cytogram and scatterplots on a blood sample from a patient with FAB M4 AML performed on a Bayer-Technicon H2 automated analyser. There are two populations of blasts, peroxidase-negative monoblasts falling into the LUC area and peroxidase-positive myeloblasts forming a large abnormal cloud in the neutrophil area; there is pseudobasophilia and thrombocytopenia. Note that the blast cluster in the LUC area extends further upwards than in the case of FAB M1 AML (as in part (c) of this figure) indicating that many of the blasts are very large. (g) Histograms and scatterplots on a blood sample from a patient with M4Eo AML performed on a Bayer-Technicon H2 automated analyser. The peroxidase scatterplot is similar to that seen in FAB M4 AML but extension into the eosinophil area is apparent; there is pseudobasophilia and thrombocytopenia. The double population shown in the red cell histogram is a result of blood transfusion, the patient having macrocytic red cells and the transfused cells being normocytic.

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TWO

IMMUNOPHENOTYPING AND CYTOGENETIC/MOLECULAR GENETIC ANALYSIS IN LEUKAEMIA AND RELATED CONDITIONS

Introduction

Cytology remains fundamental to the diagnosis and classification of haematological neoplasms. Histology supplemented by immunohistochemistry is also sometimes essential. However, in the last decade immunophenotyping and cytogenetic/molecular genetic analysis have become of crucial importance and these techniques are now widely applied. Immunophenotyping is increasingly used in all cases of acute leukaemia, myeloid as well as lymphoid. In the non-Hodgkin lymphomas (NHL) and chronic lymphoid leukaemias immunophenotyping is likewise a crucial diagnostic tool.

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Information gained from cytogenetic and molecular genetic analysis is of no less importance in haematological neoplasms – for understanding pathogenesis, making a precise diagnosis, determining prognosis, choosing treatment and developing new forms of treatment.

As immunophenotypic and genetic analysis have increased in importance, cytochemistry has become less important with its use now largely confined to acute myeloid leukaemia (AML), the myelodysplastic syndromes (MDS) and hairy cell leukaemia.

Immunophenotyping

Leukaemic cells of different types express characteristic nuclear, cytoplasmic and cell surface antigens.

This is referred to as the immunophenotype of the cell. Characterization of the immunophenotype is referred to as immunophenotyping and is achieved by means of labelled antibodies that recognize specific epitopes of cellular antigens. In general, the most useful antibodies are monoclonal antibodies (McAb) produced by hybridoma technology but, for some antigens, antisera containing polyclonal antibodies (PcAb) are better. The technique employed for immunophenotyping is usually flow cytometry although immunocytochemistry is still useful when there are few cells available for study or when there is a requirement to assess cytological features and antigen expression of the same cell. Immunohistochemistry is an essential tool when tissues require assessment, whether these be lymph nodes, trephine biopsy specimens or other tissues.

Specific surface membrane antigens of normal and leukaemic cells can be readily identified by the antibodies they bind. Using appropriate 'permeabilizing' techniques, cytoplasmic and intranuclear antigens can also be recognized. Polyclonal antibodies are raised by immunizing an animal, usually a rabbit, with normal or leukaemic cells. McAb are secreted by a clone of cells obtained by hybridizing an antibody-producing cell with a mouse myeloma cell, thus immortalizing it. McAb resulting from use of this hybridoma technology are stable and their specificity can be defined. A large number of antibodies have been characterized by a number of workshops (the International Workshops on Human Leukocyte Differentiation Antigens) and those recognizing the same antigen have been allocated to a cluster of differentiation, identified by a CD number. The CD number both identifies the specificity of the antibody and refers to the antigen. Hybridoma technology has led to the wide availability of antibodies suitable for typing leukaemic cells and has made possible standardization of techniques.

Techniques for recognizing that an antibody has bound to a cell include immunoenzymatic and immunofluorescence techniques. Immunoenzymatic techniques are applicable to fixed cells and therefore permit recognition of both surface and intracellular antigens. Either the primary antibody or a second antibody directed against antigens of the primary antibody is conjugated to an enzyme such as peroxidase or alkaline phosphatase, which produces a brown or red reaction product, visible by light microscopy. Use of both peroxidase and alkaline phosphatase conjugated to different antibodies permits the detection of coexpression of two antigens on a single cell. Immunocytochemical techniques have the advantage that the cytological characteristics of the cells can be identified (Fig. 2.1) but because such techniques are very labour intensive they have largely been replaced by flow cytometry for studying cells in the peripheral blood or a bone marrow aspirate. Immunofluorescence techniques using a microscope have also largely been superseded. Immunohistochemistry requires a reaction product visible by light microscopy; this can be achieved with various detection systems among which an antibody bound to peroxidase is most often used.

Immunofluorescence is the basis of flow cytometry immunophenotyping [1–6]. Flow cytometry has the advantage over immunocytochemistry that it is rapid and quantification of the percentage of positive cells is more precise because many more cells are evaluated (Fig. 2.2). On the one sample it is possible to determine forward light scatter (FSC) and sideways light scatter (SSC), examine the coexpression of multiple antigens and quantitate the strength of antigen expression more precisely than is possible by immunocytochemical techniques (Fig. 2.3). The antibody is bound to a fluorochrome that absorbs light then emits light of a longer wavelength, detectable at a specific relevant wavelength. A stream of cells, labelled with an antibody conjugated to a fluorescent dye, flows past a detector so that cells can be counted and their FSC, SSC and fluorescence intensity can be characterized. Forward and sideways light scatter are displayed on an arithmetic scale. FSC is mainly a function of the cell size (with some influence from the refractive index) so that it is approximately proportional to cell size. SSC detects refracted and reflected light and is influenced by the refractive index of the internal contents of the cells, such as granules. It thus indicates granularity and complexity of the cell. Cytoplasmic vacuolation affects both forward and sideways light scatter. Fluorescence intensity is usually displayed on a logarithmic scale. Non-viable cells can be identified and excluded by differential binding of specific dyes, such as propidium iodide or ethidium monoazide, to the deoxyribonucleic acid (DNA) of non-viable cells. Flow cytometric

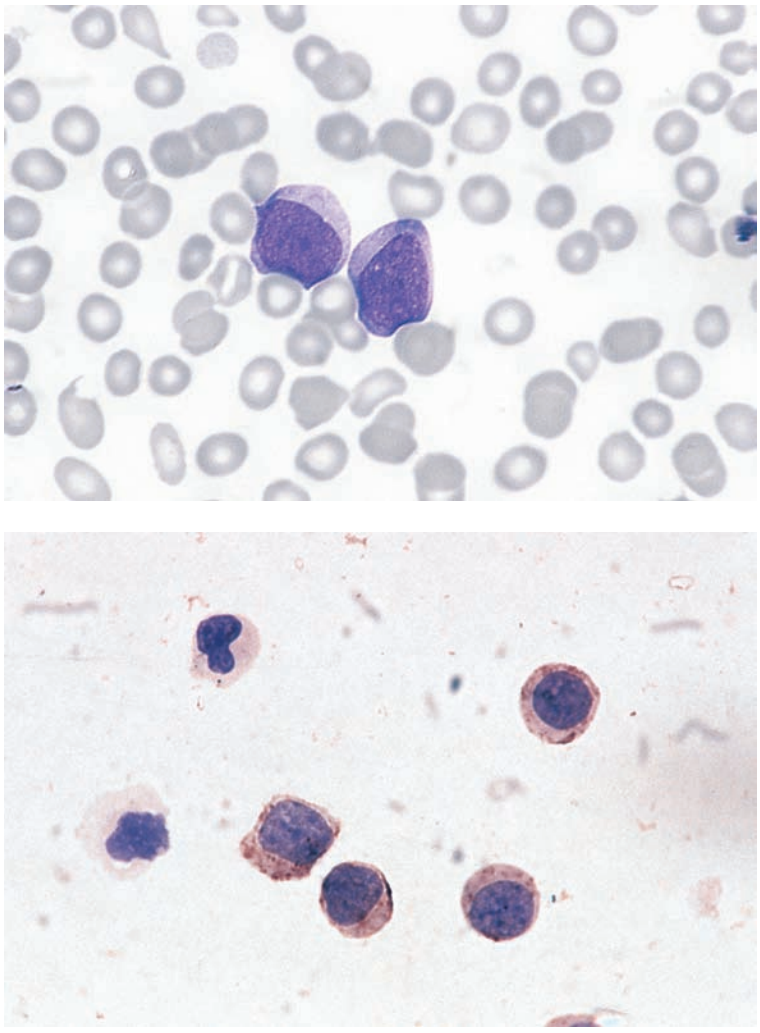


Fig. 2.1 French–American–British (FAB) M0 acute myeloid leukaemia (AML) investigated by immunophenotyping. (a) Peripheral blood (PB) film. May–Grünwald–Giemsa (MGG) ×100. (b) Cytospin preparation stained by immunoperoxidase technique with a CD13 monoclonal antibody, showing two negative lymphocytes and four positive blasts. Immunoperoxidase ×100.

techniques are applicable to either unaltered cells, in which case only surface antigens are detected, or 'permeabilized' cells, permitting detection of intracellular antigens, either cytoplasmic or nuclear. Coexpression of antigens on single cells or populations of cells can be detected by using two or more antibodies conjugated to different fluorochromes with specific emission spectra. It is possible to combine staining of a membrane antigen and of an intracellular antigen; the surface membrane staining is done first and then the cell is permeabilized for staining of the intracellular antigen. The major fluorochromes available are shown in Table 2.1. The most frequently employed are fluorescein isothiocyanate (FITC) and phycoerythrin (PE). PE and allophycocyanin (APC), which have a high quantum yield, are particularly suitable for weakly expressed antigens, whereas peridinin chlorophyll protein complex (PerCP) is better reserved for strongly expressed antigens. The optimal antibody–fluorochrome combination and the dilution must be determined within the laboratory. For example, anti-κ and anti-λ antisera should give a similar intensity of fluorescence on normal lymphocytes and, because detection of weak expression is diagnostically useful, they should not be conjugated to a fluorochrome such as PE that has a high quantum yield. Use of commercially available antibodies is preferred since this permits standardization between laboratories. Monoclonal antibodies are

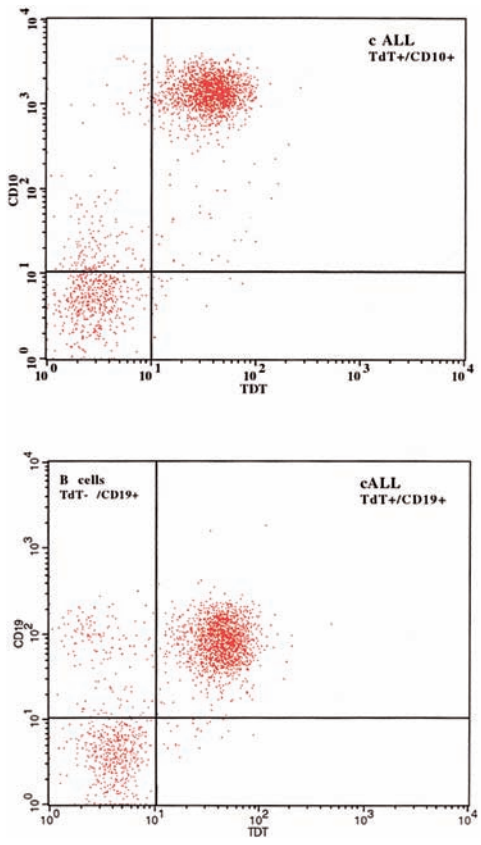


Fig. 2.2 Immunophenotyping of a case of acute lymphoblastic leukaemia (ALL) by flow cytometry with two-colour immunofluorescence: the upper scatterplot shows leukaemic cells that are positive for CD10 and terminal deoxynucleotidyl transferase (TdT), the cells that are negative for both being residual normal cells; the lower plot shows cells that are positive for both CD19 and TdT, which represent leukaemic cells, while there are two clusters of TdT-negative cells, which are positive and negative, respectively, for CD19 – these represent residual normal B cells and T cells. cALL, common ALL. (By courtesy of Mr Ricardo Morilla, London.)

generally preferred to polyclonal; however polyclonal antisera to κ and λ light chains are preferred, because of their broader specificity.

The availability of a wide range of antibodies and increasingly sophisticated instruments means that three- or four-colour analysis has now become common and six-, seven- and eight-colour flow cytometry is possible. When using multicolour analysis, it is usual to have an 'anchor' antibody that is used in every tube. It is then possible to gate

Table 2.1 The fluorochromes most often used in flow cytometric immunophenotyping and some commonly used abbreviations.

Fluorochrome	Abbreviation
Fluorescein isothiocyanate	FITC
Phycoerythrin	PE
Peridinin–chlorophyll protein complex	PerCP
Allophycocyanin	APC
Cy3 (a cyanine dye)	
Cy5 (a cyanine dye)	
Cy5.5 (a cyanine dye)	
Texas red	
Pacific blue	
Cascade yellow	
Peridinin–chlorophyll protein complex + Cy5.5 tandem conjugate	PerCP-Cy5.5
Phycoerythrin + Texas red tandem conjugate	PE-Texas red
Phycoerythrin + Cy5 tandem conjugate	PE-Cy5

on different populations, for example using CD45 (common leucocyte antigen) and SSC analysis and evaluate the expression of different antigens in relation to a specific CD45/SSC cluster. Gating is very useful when there is an admixture of normal and neoplastic cells (Fig. 2.4). Populations under evaluation can be displayed in consistent colours in different plots. CD45 expression increases as cells mature and, for cells of granulocyte lineage, SSC is greater in the case of more mature cells. It is possible to provisionally identify clusters of neutrophils, monocytes, lymphocytes and blast cells on the basis of the CD45/SSC plot. Other gating policies can also be employed. It is possible to gate on B cells, T cells, large cells (using FSC) or cells that express a disease-related phenotype (e.g. CD19-positive CD5-positive B cells in order to assess ZAP70, a prognostic marker, on the cells of chronic lymphocytic leukaemia). When gating on cells in a 'blast window' it should be noted that not all leukaemic blast cells will fall into this area (Fig. 2.5); monoblasts and the leukaemic cells of French–American–British (FAB) M3 AML usually fall largely elsewhere. All data are usually collected ungated and gating is subsequently applied so that information on any minor abnormal population is not lost. In addition, collecting all data means that normal cells can be used as internal positive and negative controls. However, in some circumstances, e.g. in assessment of minimal residual

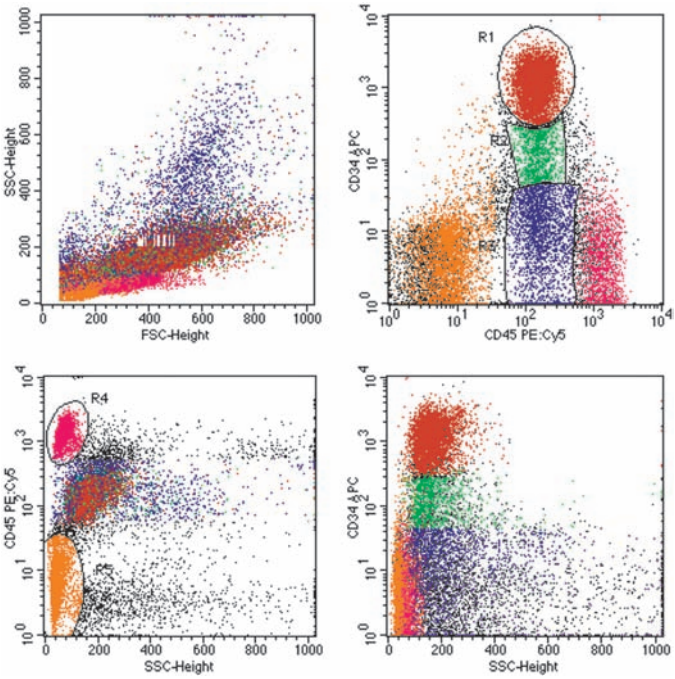


Fig. 2.3 Four-colour flow cytometric immunophenotyping showing characteristics of various types of cell. Standard analysis regions (R1–R3) are set to delineate stages of myeloid blast cell differentiation: R1, the most immature blast cells (CD34+++); R2, maturing blast cells (CD34 +); R3, mature blast cells and maturing granulocytes; R4, normal lymphocytes (based on CD45+++ expression and sideways light scatter (SSC) characteristics (SSC+); R5, nucleated red cells, which are CD45–, SSC–. These regions can be systematically applied to simultaneously assess a series of antibody combinations relevant to the classification of AML. APC, allophycocyanin; FSC, forward light scatter; PE, phycoerythrin. (By courtesy of Dr Steve Richards, Leeds.)

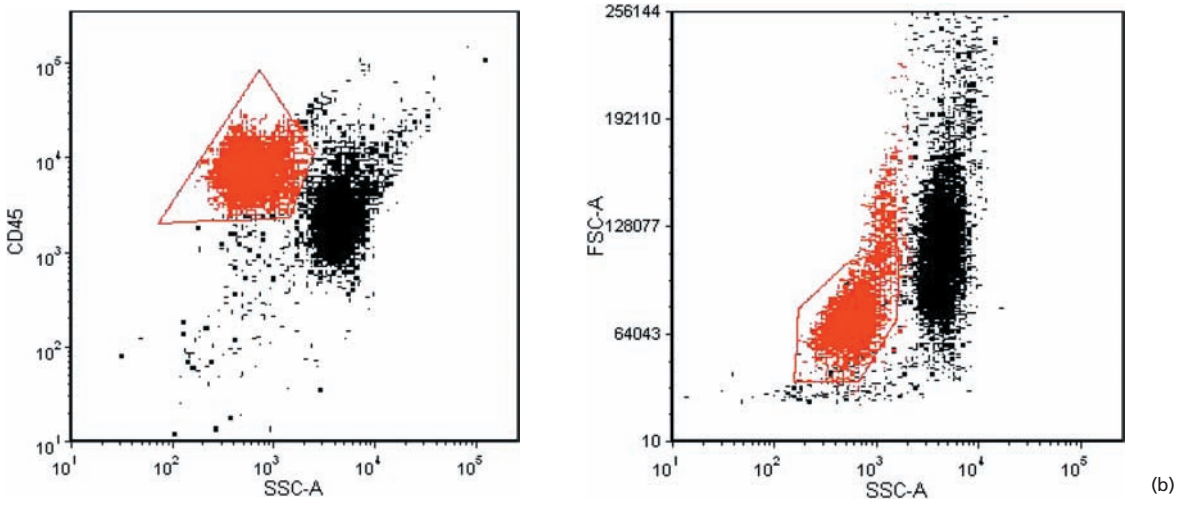
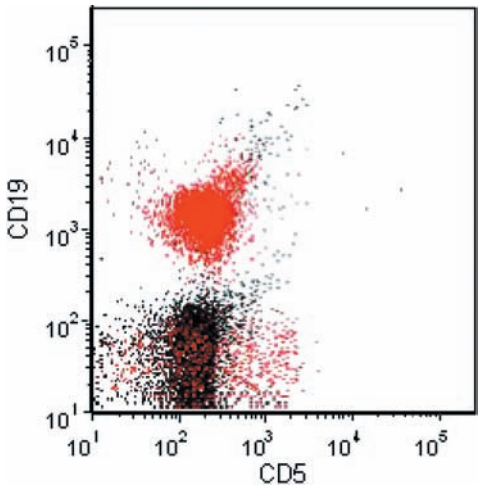
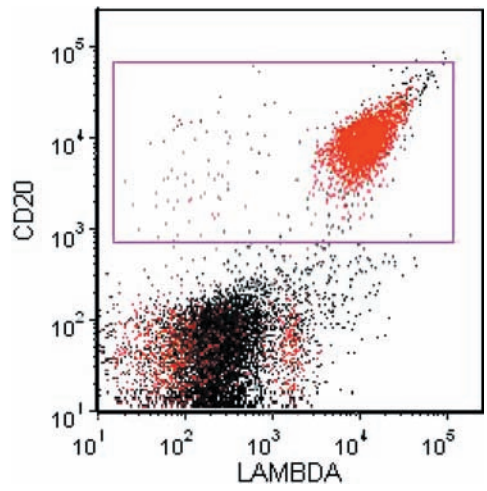


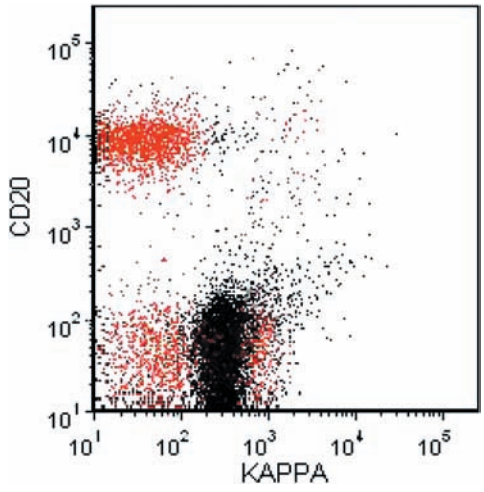
Fig. 2.4 Flow cytometric immunophenotyping in a patient with mantle cell lymphoma showing an abnormal population of B cells expressing CD19, CD5, λ and CD79b. (a, b) Plots of CD45 versus SSC and of FSC versus SSC identified two populations of cells; the cluster painted red in these plots is the same population and one or other gate has been applied to produce all the following scatter plots: (c) the gated population includes an abnormal CD5+ CD19+ population (lymphoma cells) and a CD5+ CD19– population (normal T cells, expressing CD5 more strongly than the lymphoma cells); (d, e) the CD20+ B cells are mainly expressing λ and not κ; (f) the gated population also includes some CD3+ CD4+ normal T cells; (g) most of the CD19+ B cells express CD79b (weakly); (h) there is expression of FMC7 and variable expression of CD23 but with the majority of cells being positive. (Expression of CD23 is not usual in mantle cell lymphoma.) (By courtesy of Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.) (*Continued opposite*)


CD19
CD5

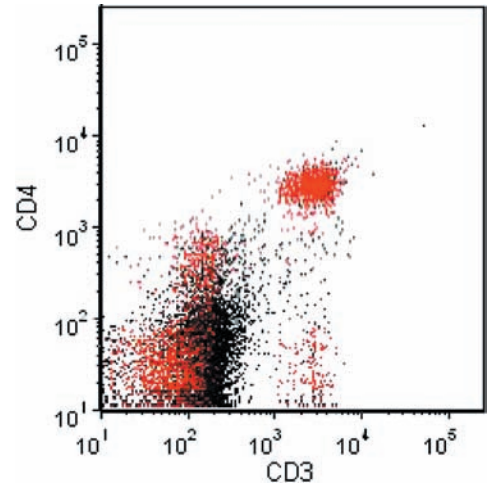
(c)


CD20
LAMBDA

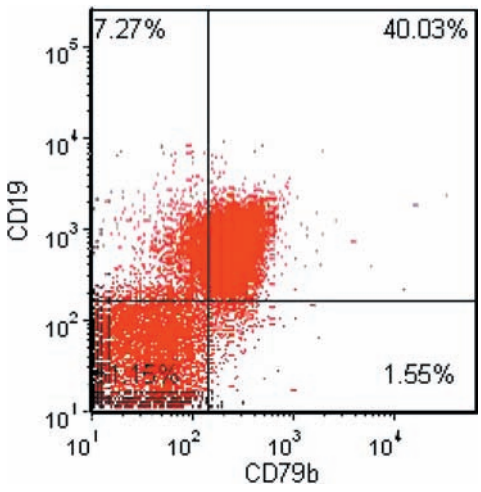
(d)


CD20
KAPPA

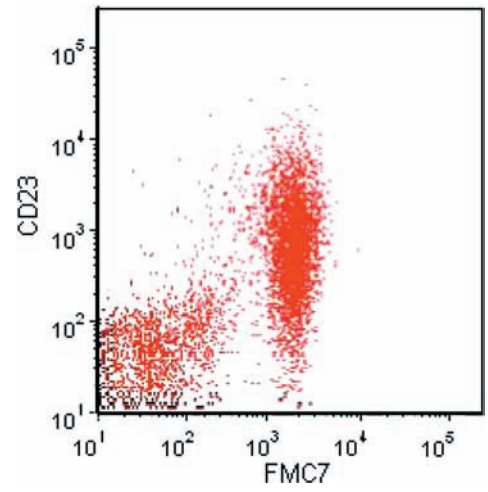
(e)


CD4
CD3

(f)


7.27%
40.03%
1.55%
CD19
CD79b

(g)


CD23
FMC7

(h)

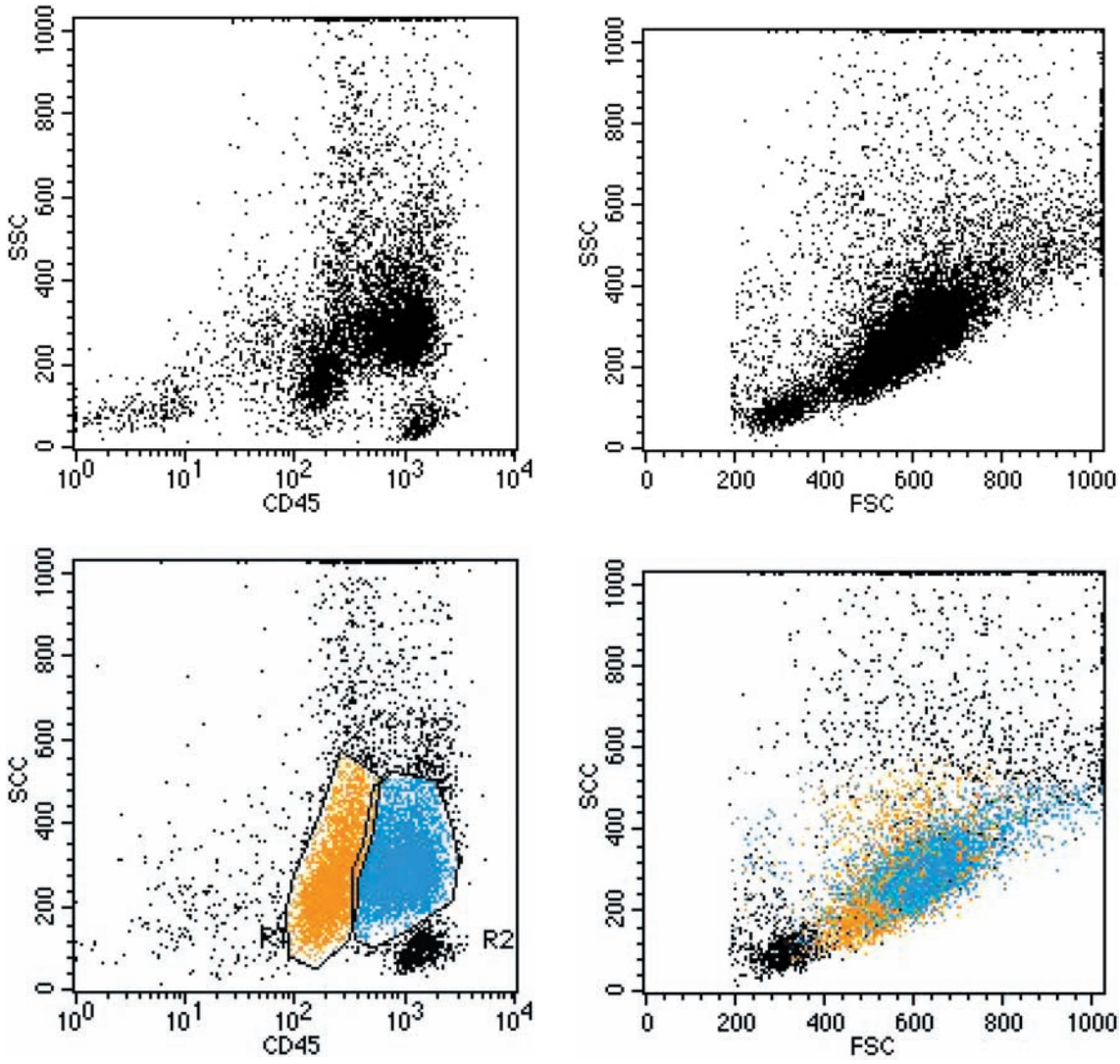


Fig. 2.5 Dot plots of flow cytometric immunophenotyping of bone marrow cells from a patient with FAB M4 AML. Gates have been placed on two cell populations apparent in the SSC/CD45 plot, one in the usual position of blast cells (orange, R1) and one in a position where monoblasts may be found (blue, R2). The two populations differ in their light-scattering properties and CD45 expression and show subtle differences in expression of myeloid antigens. The granulocyte precursor population (orange) shows weak CD45 expression with strong CD13, and is positive for CD33, CD117, HLA-DR and myeloperoxidase (MPO) (partial). There is lack of expression of other myeloid markers (CD14, CD64 (mainly negative) and lysozyme). The monocytic precursor population (blue) shows stronger CD45 expression and slightly higher SSC. This population is positive for HLA-DR, CD14, CD64 and CD33 (strong). This pattern is characteristic of different stages of monocytic lineage maturation. There is very weak expression of lysozyme and no expression of CD13, MPO or CD117. Expression of CD117 is variable in the monocyte lineage; in this case the granulocytic population is positive and the monocytic negative. The differential expression of CD14 is consistent with the specificity of this marker for monocyte differentiation and maturation. Neither population expressed the stem cell marker, CD34, the mature granulocyte marker, CD15, T-cell markers (CD2, CD3 and CD7) or B-cell markers (CD10, CD22, CD79a and cytoplasmic μ chain). CYT, cytoplasmic; FITC, fluorescein isothiocyanate (for other abbreviations see earlier captions). (By courtesy of Mr Ricardo Morilla.)

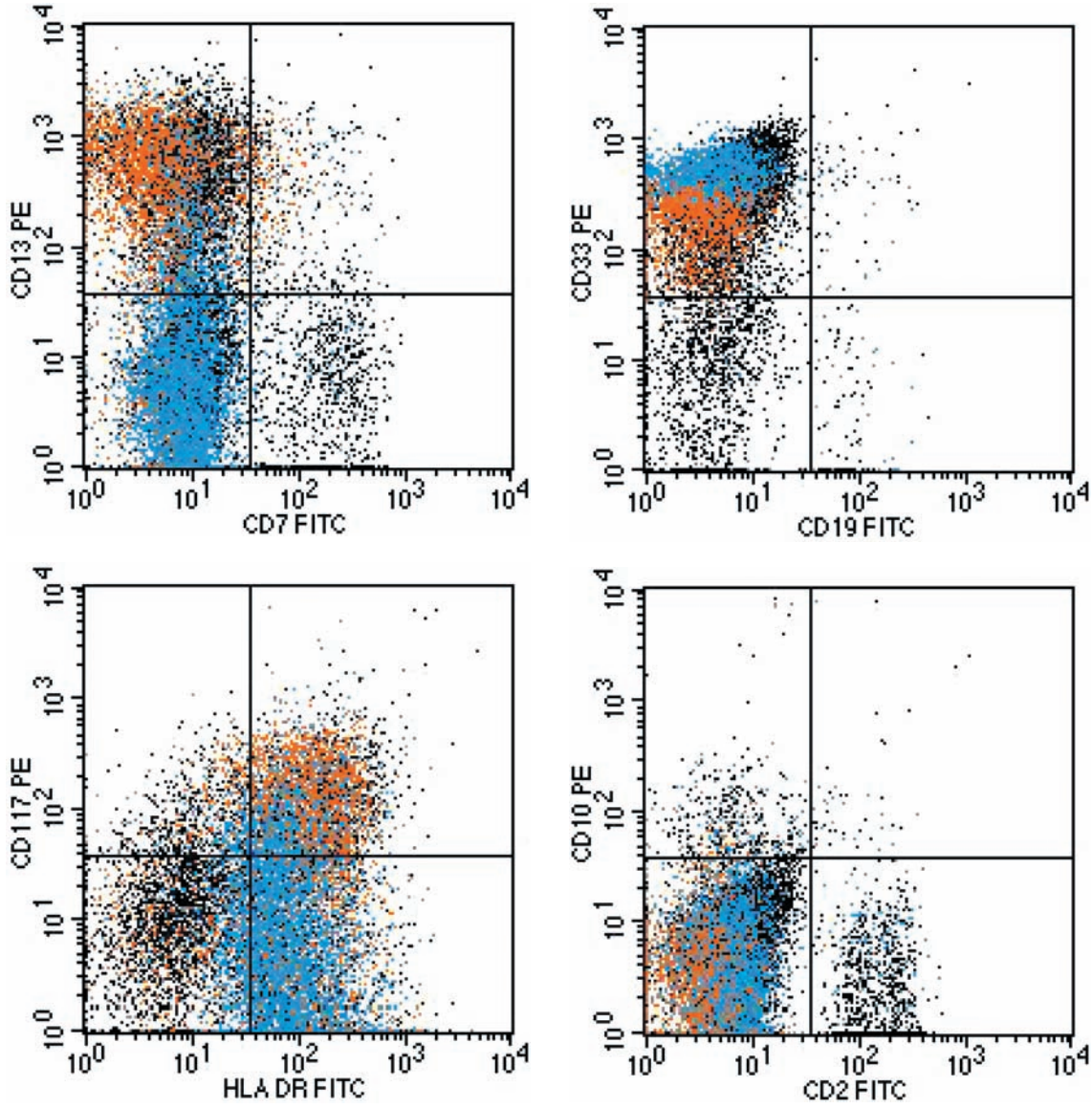


Fig. 2.5 (*Continued*)

disease, a 'live gate' is used so that a large amount of data can be collected on a specified population.

Immunophenotyping can be employed to detect oncogene products (e.g. cyclin D1), tumour suppressor gene products (e.g. p53) and the expression of genes conveying multiple drug resistance to leukaemic cells (e.g. *MDR1*, *MRP1* and *LRP*). New techniques are being developed using differentially labelled beads to capture fusion proteins or ectopically expressed or over-expressed proteins in leukaemic cell lysates, by means of monoclonal antibodies bound to the beads. This technique can indicate the likelihood of specific genetic abnormalities more rapidly than is possible with molecular techniques.

Flow cytometry immunophenotyping was initially performed on mononuclear cells that had been separated from monocytes and granulocytes on density gradients. Because the use of flow cytometry was adopted so widely it became necessary to modify

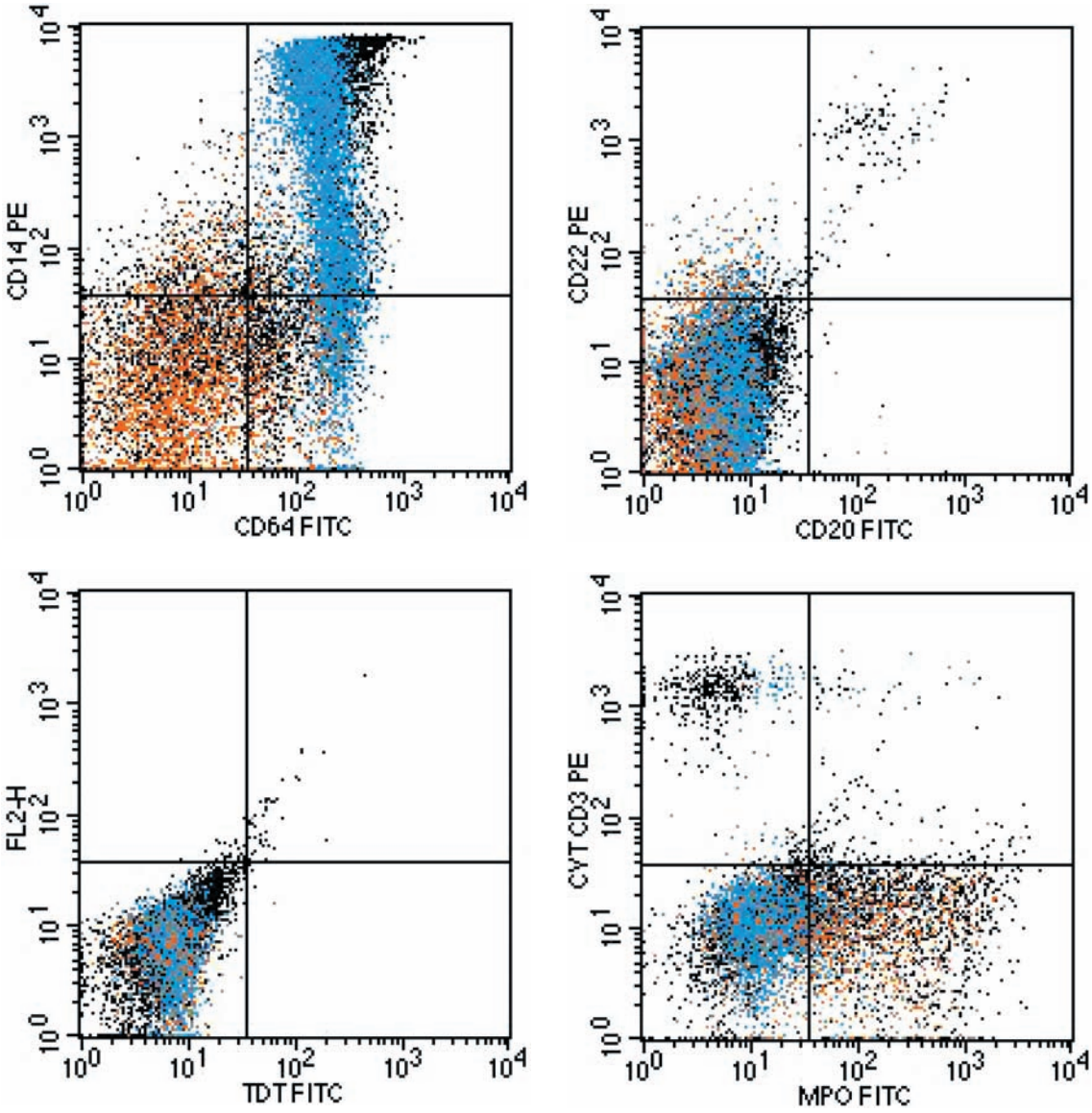
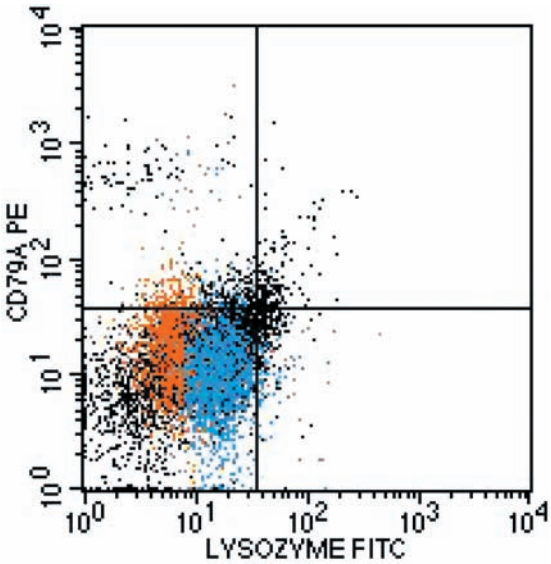


Fig. 2.5 (*Continued*)

techniques in order to cope with the work load efficiently. Analysis is therefore now generally performed on whole blood in which the red blood cells have been lysed. This latter technique has the advantage of greater speed, less loss of cells of potential interest and the retention of normal cells, such as granulocytes, that can act as an internal control for some of the antibodies employed. When antibodies to immunoglobulins or their components are being used, a wash step is needed as otherwise plasma immunoglobulins can interfere, leading to negative results. For the same reasons of efficiency, indirect labelling techniques have largely been replaced by direct labelling. In indirect labelling techniques, a primary antibody, e.g. a murine antibody, binds to an antigen. Its binding is then recognized by a second fluorochrome-labelled antibody that is directed at murine antigens. In direct labelling



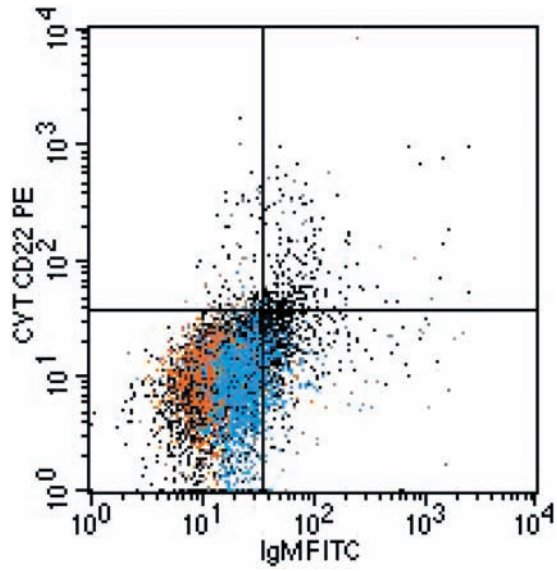


Fig. 2.5 (*Continued*)

techniques, the primary antibody is directly labelled with the fluorochrome. Direct labelling is not only less labour intensive but also permits the study of multiple antigens on the same population of cells by the use of different antibodies bound to different fluorochromes.

Assessment of flow cytometry results should incorporate an evaluation of the strength of expression of each antigen, since this is often of diagnostic importance. Comparison can be made with an isotype control – i.e. an antibody of the same isotype from the same species of animal directed at an irrelevant antigen – or with a negative control, using the binding of an antibody to cells that do not express the relevant antigen (Fig. 2.6). Controls permit correction for non-specific binding. Non-specific binding is more common with cells showing monocytic differentiation. It is also necessary to be aware of autofluorescence, which is particularly a characteristic of acute promyelocytic leukaemia. Fluorescence intensity is determined by the fluorochrome used, the strength of binding and the number of epitopes carried on a cell. Immunophenotyping laboratories often use 'dim' and 'bright' to refer to fluorescence intensity. As a broad approximation, signals between 10^0 and 10^1 can be regarded as negative, between 10^1 and 10^2 as weak

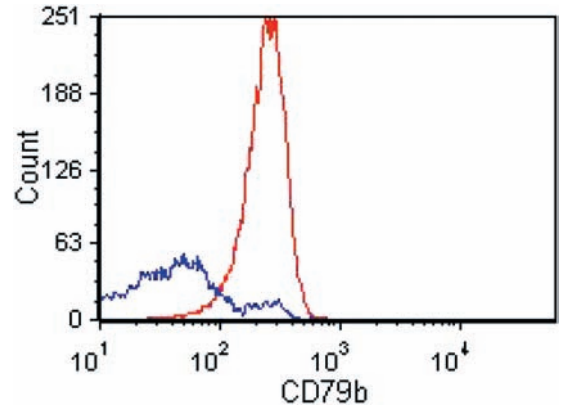


Fig. 2.6 Flow cytometry histogram showing the number of signals plotted against the intensity of the fluorescent signal at the selected wave length for CD79b (red) and a negative control (blue). These are two populations of cells in the same tube captured in different gates, red representing CD5+ CD19+ B cells and blue representing CD5+ CD19– T cells. A threshold at 10^2 would give the best separation between negative and positive for CD79b, which is expressed weakly by these B cells. (By courtesy of Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

(+), between 10^2 and 10^3 as moderate (++) and between 10^3 and 10^4 as strong (+++); however, this interpretation has to be modified, depending on the signal strength of the isotype or negative control. Calibrating beads can be used to attempt to standardize assessment of strength of expression. It is acceptable laboratory practice to omit an isotype control and use unstained cells to detect autofluorescence and use negative results on other samples in the batch as a negative control. Positive controls may also be employed but if there are normal cells in the sample they can serve as an internal positive control obviating the need for a control; similarly, if significant numbers of analyses are performed in one day then laboratories often omit a positive control since it is likely that the antigen being analysed will be expressed by one or other specimen in that day's work.

An artefact that should be recognized on flow cytometric immunophenotyping is the presence of a second cluster of cells with a higher FSC that represents doublets of cells; the signal for expression of various antigens appears to be increased.

Immunophenotyping should be performed with as little delay as possible; this can be particularly important in the case of neoplasms with a high rate of cell turnover, such as Burkitt lymphoma, since viability of cells on storage may be poor [6]. The presence of dead or dying cells leads to non-specific binding. Non-specific binding can also be due to expression of Fc receptors, e.g. by cells of monocyte lineage; this can be prevented by pre-incubation with immune sera to block the receptors. Samples anticoagulated with ethylene diamine tetra-acetic acid (EDTA) are generally satisfactory, although light-scattering characteristics of cells are more stable if the sample is taken into preservative-free heparin.

Those performing flow cytometric analysis and interpreting the results should ask themselves the following questions: What is the lineage of an identified population of cells? Are the cells mature or immature? Are they normal or abnormal? Is a differential diagnosis or a specific diagnosis suggested by the results? Are there further flow cytometric (or other) analyses that should be done on the specimen in the light of the initial results? Comparison should be made with a stained film from the sample analysed to ensure that cells of interest were present in the sample and have been identified by flow cytometry. Results presented should be the result of a visual appraisal that leads to recognition of normal or abnormal populations of cells and an assessment of which antigens they are expressing and the strength of expression. The gating policy followed should be stated early in the report. It is much less satisfactory to express results as the percentage of cells positive for a given antigen using an arbitrary cut-off point of 10% or 20% positive cells to denote positivity. This may lead to a minor abnormal population being missed and gives no clear information about coexpression of antigens by a single population. Expression of results as a percentage and an absolute count is, however, appropriate for quantifying B-cell or T-cell subsets in patients with suspected inherited or acquired immune deficiency. Results should generally be expressed in terms of a CD number (when applicable) but when different antibodies within a cluster are known to differ in their reactivity the clone name should also be given.

Samples for immunophenotypic analysis should arrive in the laboratory accompanied by all relevant information including the nature of the sample (e.g. peripheral blood or bone marrow), age, gender and identifying details of the patient, clinical history, physical findings, blood count results and suspected diagnosis. This information is essential both to select the initial panel of antibodies (particularly important for specimens of low cellularity) and to interpret the results.

In an individual patient, the role of immunophenotyping may be: (i) confirming a diagnosis; (ii) identifying prognostic differences within a diagnostic category; (iii) staging a disease; and (iv) detecting an aberrant immunophenotype that can be used for monitoring minimal residual disease. Confirming clonality, most readily done for B cells, is an important function, which can contribute to diagnosis and also to staging (when clonal cells are identified in specific tissues). The role of immunophenotyping is not 'making a diagnosis'. Immunophenotyping is just one part of a jigsaw that has to be fitted together. The immunophenotype should never be considered in isolation. It is essential to consider all information that is available – cytology, histology, immunophenotype and genetic characteristics – in order to make an accurate and precise diagnosis. Nevertheless, there are some abnormal immunophenotypes that are so distinctive that they do indicate a specific diagnosis.

Immunophenotyping is essential for the diagnosis of B- or T-lineage acute lymphoblastic leukaemia (ALL). In AML immunophenotyping is essential in the diagnosis of FAB M0 and M7 categories and AML with an early erythroid phenotype, subtypes of AML that can be confused with ALL if the diagnosis has to be based on cytology alone. Immunophenotyping is essential for the identification of mixed phenotype acute leukaemia ('biphenotypic leukaemia' and 'bilineal leukaemia') and undifferentiated stem cell leukaemia (see below). The immunophenotype can form the sole basis of a classification of AML [7] but this is not recommended since a single specific type of AML may fall into two different immunophenotypic categories. More satisfactory classifications are based on integration of all information, as is done in the World Health Organization (WHO) classification.

Immunophenotyping is equally important in lymphoproliferative disorders, both for confirming a suspicion of a neoplastic condition and for distinguishing between different types of lymphoid leukaemia and lymphoma. With the introduction of more intensive treatment, monitoring minimal residual disease has become more important, both in chronic lymphocytic leukaemia (CLL) and in multiple myeloma.

Approaches to the selection of an antibody panel differ. One approach is to have a relatively small primary panel, which is chosen according to the provisional diagnosis, and a secondary panel, which is applied selectively, depending on the results with the first panel. This is the only approach that can be followed if there is only limited material available and it has the advantage that it is economical with reagents. However, it does require the application of judgement and, if the provisional diagnosis or the initial interpretation is wrong, an inappropriate panel of antibodies may be applied. An alternative approach is to use a general comprehensive panel. This means that a large amount of information is gathered speedily without the need for decisions to be made with regard to the choice of antibodies. Reagent costs are necessarily higher but there is a greater probability that all necessary data will be collected.

Problems and pitfalls in immunophenotyping

A bone marrow aspirate may show no immunophenotypic abnormality despite bone marrow infiltration, as a result of the necessarily random nature of sampling or because reactive fibrosis prevents neoplastic cells from being aspirated. For this reason, a trephine biopsy supplemented by immunohistochemistry is important when sampling error and failure to aspirate neoplastic cells are known to be likely, e.g. in follicular lymphoma or systemic mastocytosis. Similarly, and for the same reasons, flow cytometry immunophenotyping can underestimate the disease burden, e.g. in multiple myeloma.

Light chain restriction is usually taken as evidence of neoplasia but it should be noted that this is not always so, e.g. it has been observed in florid reactive follicular hyperplasia [8]. An abnormal T-cell phenotype is not specific for T-cell neoplasia, absence or down-regulation of some antigens (including CD7) being seen, for example, in infectious mononucleosis, reactive dermatoses and inflammatory conditions [9].

Errors in interpretation often result from a failure to correlate flow cytometry results with clinical and haematological features. This can lead to use of an antibody panel that is either too restricted or inappropriate or to gating on the wrong cell cluster. Serious errors include mistaking haematogones for leukaemic lymphoblasts or mistaking immature erythroid cells (e.g. in megaloblastic anaemia or congenital dyserythropoietic anaemia) for neoplastic cells of erythroleukaemia [10].

Immunophenotyping in acute leukaemia

Immunophenotyping is indicated in all cases of acute leukaemia that are not obviously myeloid, in order to make a positive diagnosis of ALL and recognize all cases of FAB M0 and M7 AML. A possible further indication is the recognition of an immunophenotype that is likely to indicate a specific subtype of acute leukaemia or that will be useful for subsequent monitoring of minimal residual disease.

Several standard panels for the initial phenotyping of acute leukaemia have been proposed [11–18]. The more important McAb and PcAb used in acute leukaemia diagnosis and classification are shown in Table 2.2 [19] and the panels recommended by the European Group for the Immunological Characterization of Leukemias (EGIL) [15], the US–Canadian Consensus Group [17], the British Committee for Standards in Haematology (BCSH) [18], the European LeukaemiaNet and the WHO expert groups in Tables 2.3 to 2.6.

Table 2.2 Monoclonal (or polyclonal) antibodies useful in the diagnosis and classification of acute leukaemia.

Cluster of differentiation (CD) or other specificity*	Specificity within haemopoietic lineage
Antibodies identifying antigens expressed mainly in haemopoietic precursors	
CD34	B-lineage and T-lineage precursors, myeloid progenitors, blast cells of 60–70% of cases of B-lineage ALL; blast cells of <10% of cases of T-lineage ALL; blast cells in most cases of AML
Anti-HLA-DR	Major histocompatibility complex, class II antigens; expressed on B lymphocytes and B-lymphocyte progenitors, activated T lymphocytes, monocytes and their precursors, myeloid precursors, blast cells of B-lineage ALL and of a small minority of cases of T-lineage ALL, blast cells of most cases of AML
Anti-terminal deoxynucleotidyl transferase (TdT)	Blast cells of ALL (stronger in B lineage than T-lineage blasts), more weakly expressed in blasts in 10–20% of AML
Antibodies identifying antigens expressed in all leucocytes	
CD45	Common leucocyte antigen; expressed on normal leucocytes, in 90% of cases of B-lineage ALL and in almost all AML and T-lineage ALL; use of CD45 permits gating on blast cells which express CD45 and have low sideways light scatter – however note that leukaemic blast cells, particularly B-lineage lymphoblasts, may fail to express CD45 or may express it weakly; cells of neutrophil lineage show increased CD45 expression with maturation; monocytes and eosinophils show stronger expression than neutrophils
Antibodies identifying antigens expressed mainly by B cells	
CD10	Common ALL antigen; expressed on a subset of B-cell progenitors, blast cells of about 90% of cases of B-lineage ALL, more weakly expressed in some T-lineage ALL (*c.* 15–20%), expressed in most follicular lymphomas and some multiple myeloma, expressed by neutrophils
CD19	B lymphocytes and B-lymphocyte precursors, blast cells of B-lineage ALL; expressed in some cases of AML, particularly AML associated with t(8;21)
CD20	B lymphocytes, some B-lymphocyte precursors, blast cells in about 40% of cases of B-lineage ALL
CD22	B lineage: as a surface antigen in B lymphocytes, as a cytoplasmic antigen in B-lymphocyte precursors, as a surface antigen in some B-lineage ALL and as a cytoplasmic antigen in *c.* 98%
CD24	B lymphocytes and precursors, blast cells of B-lineage ALL (at least 90% of cases), activated T lymphocytes, granulocytes (neutrophils and eosinophils)
CD79a	Part of the B-cell receptor; expressed by B cells and their precursors and plasma cells; aberrantly expressed in some cases of T-lineage ALL and AML
CD79b	Part of the B-cell receptor; expressed by most normal and abnormal B cells and late B-cell precursors (from the pre-B cell onwards) but not expressed in chronic lymphocytic leukaemia
Anti-immunoglobulin and anti-γ, α, μ, δ immunoglobulin heavy chains	Surface membrane expression in B cells (SmIg), cytoplasmic expression in pre-B cells (cμ chain) and in late B lymphocytes and plasma cells (cIg)
Anti-κ, λ (anti-immunoglobulin light chains)	Surface membrane expression in B lymphocytes and cytoplasmic expression in late B lymphocytes and plasma cells
Antibodies identifying antigens expressed mainly in T cells	
CD1a	Cortical thymocytes, blast cells of about 20% of T-lineage ALL, subset of B cells, Langerhans cells
CD2	Cortical and late thymocytes, mature T lymphocytes, most NK cells, blast cells of *c.* 80% of T-lineage ALL, leukaemias of mature T cells; expressed in about 10% of cases of AML, particularly FAB M3 and M4Eo AML; expressed by neoplastic mast cells

Table 2.2 (*Continued*)

Cluster of differentiation (CD) or other specificity*	Specificity within haemopoietic lineage
CD3	Part of the TCR complex; membrane antigen in late thymocytes and mature T lymphocytes, blast cells of *c.* 25% of T-lineage ALL and in leukaemias of mature T cells, cytoplasmic expression is found in the majority of thymocytes and blast cell of T-lineage ALL
CD4	Cortical thymocytes (coexpressed with CD8), subset of late thymocytes, subset of mature T cells, some leukaemias of mature T cells (see Table 6.16), immature myeloid cells, monocytes (weaker than on T cells) and eosinophils; expressed in some cases of AML, particularly when there is monocytic differentiation; expressed by blastic plasmacytoid dendritic cell neoplasm
CD5	Cortical and late thymocytes, some early thymocytes, T lymphocytes, blast cells of 90–95% of cases of T-lineage ALL, small subset of B lymphocytes, some leukaemias and lymphomas of mature B cells and mature T cells (see Tables 6.7 and 6.16)
CD7	Thymocytes, majority of mature T cells, NK cells, blast cells of T-lineage ALL, subset of immature myeloid cells, blast cells of 5–15% of AML, some leukaemias of mature T cells (see Table 6.16)
CD8	Cortical thymocytes (coexpressed with CD4), subset of late thymocytes, subset of mature T cells, some cases of T-lineage ALL, some leukaemias of mature T cells (see Table 6.16)
Anti-TCR αβ	Most circulating T lymphocytes and some T-lineage ALL
Anti-TCR γδ	Small subset of circulating T lymphocytes and blast cells of some T-lineage ALL; most cases of hepatosplenic T-cell lymphoma
Antibodies identifying antigens expressed mainly in myeloid cells	
CD11b	C3bi receptor; expressed on mature monocytes, cells of neutrophil lineage with expression increasing with maturation – however mature neutrophils show weaker expression than mature monocytes; blast cells of most monocytic and some granulocytic leukaemias, macrophages, NK cells
CD13	Pan-myeloid, most strongly expressed on blast cells and neutrophils: membrane expression in blast cells of *c.* 80% of cases of AML, cytoplasmic expression in a higher proportion; expressed in 20–35% of cases of ALL
CD14	Monocytes, macrophages, granulocytes to a lesser extent, blast cells of monocytic and some granulocytic leukaemias (particularly FAB types M4 and M5b)
CD15	Maturing myeloid cells (granulocytic more than monocytic); expressed in 50% of cases of AML; aberrantly expressed in 5–10% of cases of ALL, particularly B-lineage ALL with t(4;11) but also some T-ALL
CD16	Neutrophils and NK cells, weakly expressed on monocytes
CD33	Myeloid progenitors and some maturing myeloid cells (myeloblasts, promyelocytes, myelocytes, monocytes – cells of neutrophil lineage express somewhat less CD33 as they mature and monocytes express CD33 more strongly than neutrophils), blast cells of *c.* 80% of cases of AML and 20–35% of cases of ALL
CD36	Platelet glycoprotein IV; expressed on erythroblasts and progenitors, monocytes, macrophages, megakaryoblasts, megakaryocytes and platelets; in AML expressed mainly in FAB types M5, M6 and M7; useful for identifying erythroid cells if megakaryocyte and other myeloid markers are negative
CD41	Platelet glycoprotein IIb/IIIa complex (CD41a) and platelet glycoprotein IIb (CD41b); expressed on megakaryoblasts, megakaryocytes, platelets
CD42a	Platelet glycoprotein IX; expressed on megakaryoblasts, megakaryocytes, platelets
CD42b	Platelet glycoprotein Ibα; expressed on megakaryoblasts, megakaryocytes, platelets
CD61	Platelet glycoprotein IIIa; expressed on megakaryoblasts, megakaryocytes, platelets
CD64	Monocytes, macrophages, activated granulocytes; expressed preferentially in AML with monocytic differentiation

Continued

Table 2.2 (*Continued*)

Cluster of differentiation (CD) or other specificity*	Specificity within haemopoietic lineage
CD65, CD65s	Cells of granulocytic and monocytic lineages (weaker expression on monocytes); expressed in most cases of AML, aberrantly expressed in 5–10% of cases of ALL, particularly ALL with t(4;11)
CD66c	Granulocytes and their precursors; monocytes; some B-lineage ALL, particularly those with high hyperdiploidy or *BCR-ABL1*
CD71	Erythroid cells of all stages of maturation but not lineage specific; most strongly expressed by the earliest cells; expressed by immature or activated cells of other lineages
CD117	Stem cell factor receptor, KIT: haemopoietic precursors, myeloblasts, primitive erythroid cells, some megakaryoblasts, mast cells, blasts of AML, neoplastic cells in some cases of multiple myeloma; some small cell carcinoma of the lung
Anti-myeloperoxidase (MPO)	Myeloid cells (granulocytic more than monocytic) – cytoplasmic expression
Anti-lactoferrin	A marker of maturation in the neutrophil lineage so can help to distinguish leukaemic cells from residual normal cells – cytoplasmic expression
CD235a (anti-glycophorin A) or CD236R (anti-glycophorin C)	Erythroid cells

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; c, cytoplasmic; CD, cluster of differentiation; FAB, French–American–British (leukaemia classification); Ig, immunoglobulin; NK, natural killer; TCR, T-cell receptor; Sm, surface membrane.
* For a complete list of the specificities of monoclonal antibodies assigned to each CD category see reference 19.

Table 2.3 Panel of antibodies recommended by the European Group for the Immunological Characterization of Leukemias (EGIL) for the diagnosis and classification of acute leukaemia [15].

First panel	
B lymphoid	CD19, cCD22, CD79a, CD10
T lymphoid	cCD3, CD2, CD7
Myeloid	Anti-MPO, CD13, CD33, CD65, CD117
Non-lineage specific	Anti-TdT, CD34, anti-HLA-DR
Second panel	
If B lineage	Anti-cμ, anti-κ, anti-λ, CD20, CD24
If T lineage	CD1a, SmCD3, CD4, CD5, CD8, anti-TCR αβ, anti-TCR γδ
If myeloid	Anti-lysozyme, CD14, CD15, CD41, CD61, CD64, CD235a (anti-glycophorin A)

c, cytoplasmic; CD, cluster of differentiation; MPO, myeloperoxidase; Sm, surface membrane; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.

Table 2.4 Panel of antibodies recommended by the US–Canadian Consensus Group for the diagnosis and classification of acute leukaemia [17].

Core panel	
B lymphoid	CD10, CD19, anti-κ, anti-λ
T lymphoid	CD2, CD5, CD7
Myeloid	CD13, CD14, CD33
Non-lineage related	CD34, anti-HLA-DR
Supplementary panel	
B lymphoid	CD20, Sm/cCD22
T lymphoid	CD1a, Sm/cCD3, CD4, CD8
Myeloid	CD15, CD16, CD41, CD42b, CD61, CD64, CD71, CD117, anti-MPO, CD235a (anti-glycophorin A)
Non-lineage related	CD38, anti-TdT

c, cytoplasmic; CD, cluster of differentiation; MPO, myeloperoxidase; Sm, surface membrane; TdT, terminal deoxynucleotidyl transferase.

Table 2.5 Panel of antibodies recommended by the British Committee for Standards in Haematology (BCSH) for the diagnosis and classification of acute leukaemia [18].

Primary panel	
B lymphoid	CD19, cCD22, cCD79a, CD10
T lymphoid	cCD3, CD2
Myeloid	CD13, CD117, anti-MPO
Not lineage restricted	Anti-TdT
Supplementary panel for selective application	
B lymphoid	Anti-cμ, anti-SmIg, CD138
T lymphoid	CD7
Myeloid	CD33, CD41, CD42, CD61, CD235a (anti-glycophorin A)
Not lineage restricted	CD45
Non-haemopoietic	Antibodies for the detection of small round cell tumours of childhood and other non-haemopoietic neoplasms
Optional and potentially useful	
Myeloid	Anti-lysozyme, CD14, CD36, anti-PML (McAb PL-M3), anti-HLA-DR (for negativity in FAB M3 AML)
B lymphoid	CD15 and 7.1/NG2* (for *MLL*-rearranged ALL)
T lymphoid	Anti-TCR αβ, anti-TCR γδ

ALL, acute lymphoblastic leukaemia; c, cytoplasmic; CD, cluster of differentiation; FAB, French–American–British (leukaemia classification); McAb, monoclonal antibody; MPO, myeloperoxidase; PML, nuclear protein encoded by the *PML* gene; SmIg, surface membrane immunoglobulin; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.
* 7.1/NG2 is a monoclonal antibody recognizing chondroitin sulphate.

Table 2.6 Panel of antibodies recommended by the European LeukemiaNet for the diagnosis and classification of acute leukaemia.

Initial panel for quick orientation or for samples with a low cell count
cCD3, anti-MPO, cCD79a, anti-TdT
CD7, CD2, CD10, CD19, CD22 (s or c), anti-sIg, CD13, CD33, CD34
CD45 for gating purposes
Panel for sublineage classification and definition of clinical entities
Anti-HLA-DR, CD1a, CD4, CD5, CD8, CD3 (s), anti-IgM (c), CD14, CD117, CD56, CD65, CD41 or CD61, erythroid marker such as CD235a (glycophorin A) or CD36

c, cytoplasmic; CD, cluster of differentiation; Ig, immunoglobulin; MPO, myeloperoxidase; s, surface membrane; TdT, terminal deoxynucleotidyl transferase.

As the detection of minimal residual disease (MRD) has become more important in the management of patients with acute leukaemia [20], a new role for immunophenotyping has emerged, immunophenotyping providing an alternative to genetic analysis for this purpose. A larger panel of antibodies is needed than when immunophenotyping is used only for diagnosis. The leukaemia-associated phenotype may be aberrant (Figs 2.7 and 2.8), very uncommon among normal haemopoietic and lymphoid cells or not normally found in the bone marrow. Clinically relevant information can be obtained by monitoring MRD after induction of remission, at various stages during treatment and during post-treatment follow-up. Multiparameter flow cytometry with a large panel of antibodies permits detection of a leukaemia-related immunophenotype in more than 90% of childhood cases of acute leukaemia [21,22]. The immunophenotype may change at relapse, either because of a true change in antigen expression or because there is an alteration in the proportions of different subsets of leukaemic blast cells. For this reason it is desirable, when possible, to identify two or more leukaemia-associated phenotypes for monitoring purposes. Abnormalities that can be utilized for detection of a leukaemia-associated immunophenotype include: (i) aberrant expression, i.e. expression of an antigen inappropriate to the lineage; (ii) under- or over-expression of an antigen, e.g. absence or down-regulation of CD45 or lack of expression of CD13, CD33 or human leucocyte antigen DR (HLA-DR) by

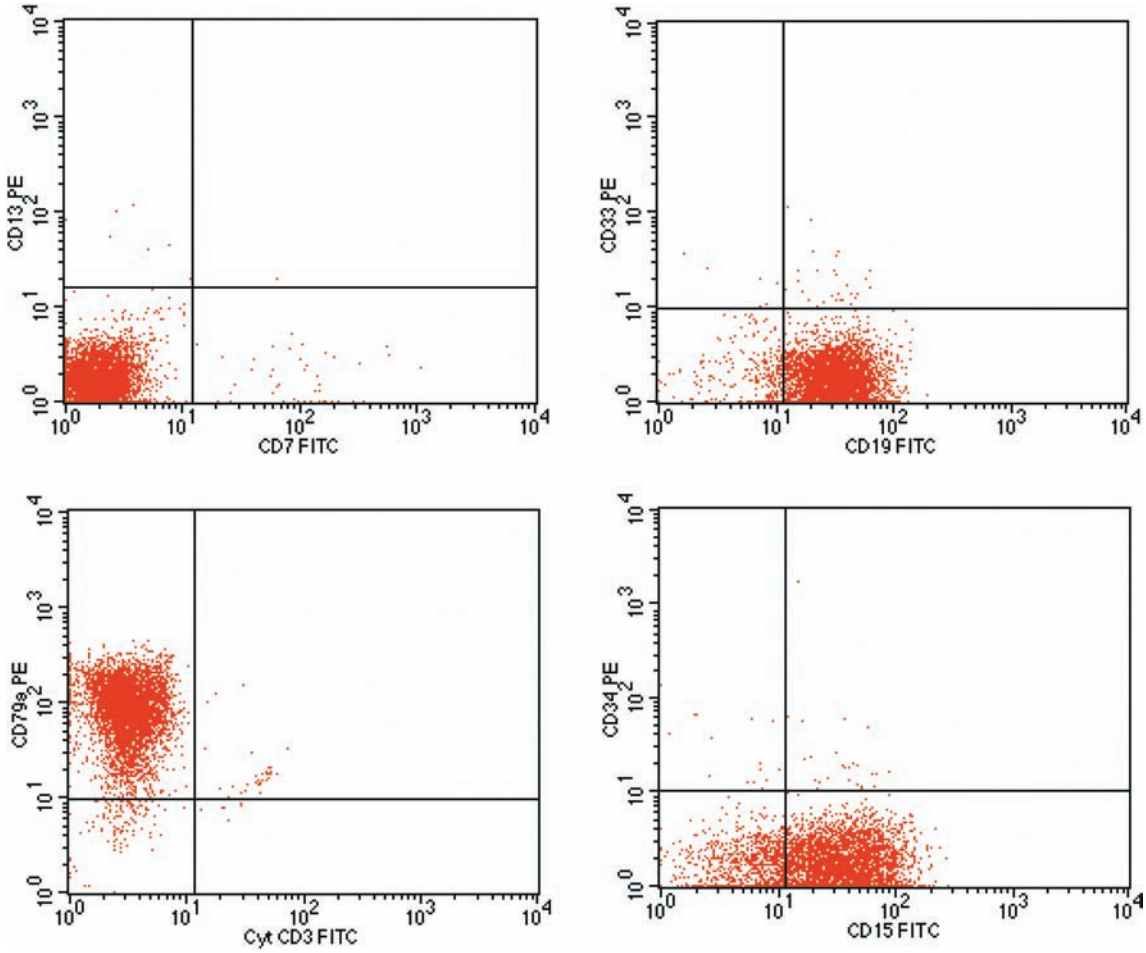


Fig. 2.7 Flow cytometric immunophenotyping in ALL showing aberrant antigen expression that could be used for monitoring minimal residual disease (MRD). The leukaemic B lymphoblasts are appropriately expressing CD19 and CD79a but are also showing aberrant expression of CD15, a myeloid-associated antigen. (By courtesy of Mr Ricardo Morilla and Professor Daniel Catovsky, London.)

myeloid cells; (iii) asynchronous expression, e.g. coexpression of CD3 with either CD34 or terminal deoxynucleotidyl transferase (TdT) or, in the case of myeloid cells, coexpression of CD11b, CD14 or CD15 with CD34; and (iv) expression of an antigen inappropriate to a tissue, e.g. CD1a on bone marrow T-lineage cells.

In ALL, MRD has been found to be of prognostic significance in both B- and T-lineage disease, in both children and adults, and following both chemotherapy and stem cell transplantation. Its assessment is now influencing patient management, with treatment being reduced in some children with favourable findings [12,13,23–25]. In ALL, MRD at day 8, at day 29 and at end of consolidation are all independent prognostic indicators [23]. MRD is also of prognostic significance in AML [24] although the significance differs between different cytogenetic/molecular genetic subtypes. Monitoring MRD during treatment permits an adjusted estimate of the risk of relapse and reduction or augmentation of therapy, depending on the new estimate of risk group [26]. The reappearance of evidence of MRD in AML may be an indication for therapeutic intervention, e.g. for donor leucocyte infusion in a patient who has had a stem cell transplant or for alteration of treatment in acute promyelocytic leukaemia.

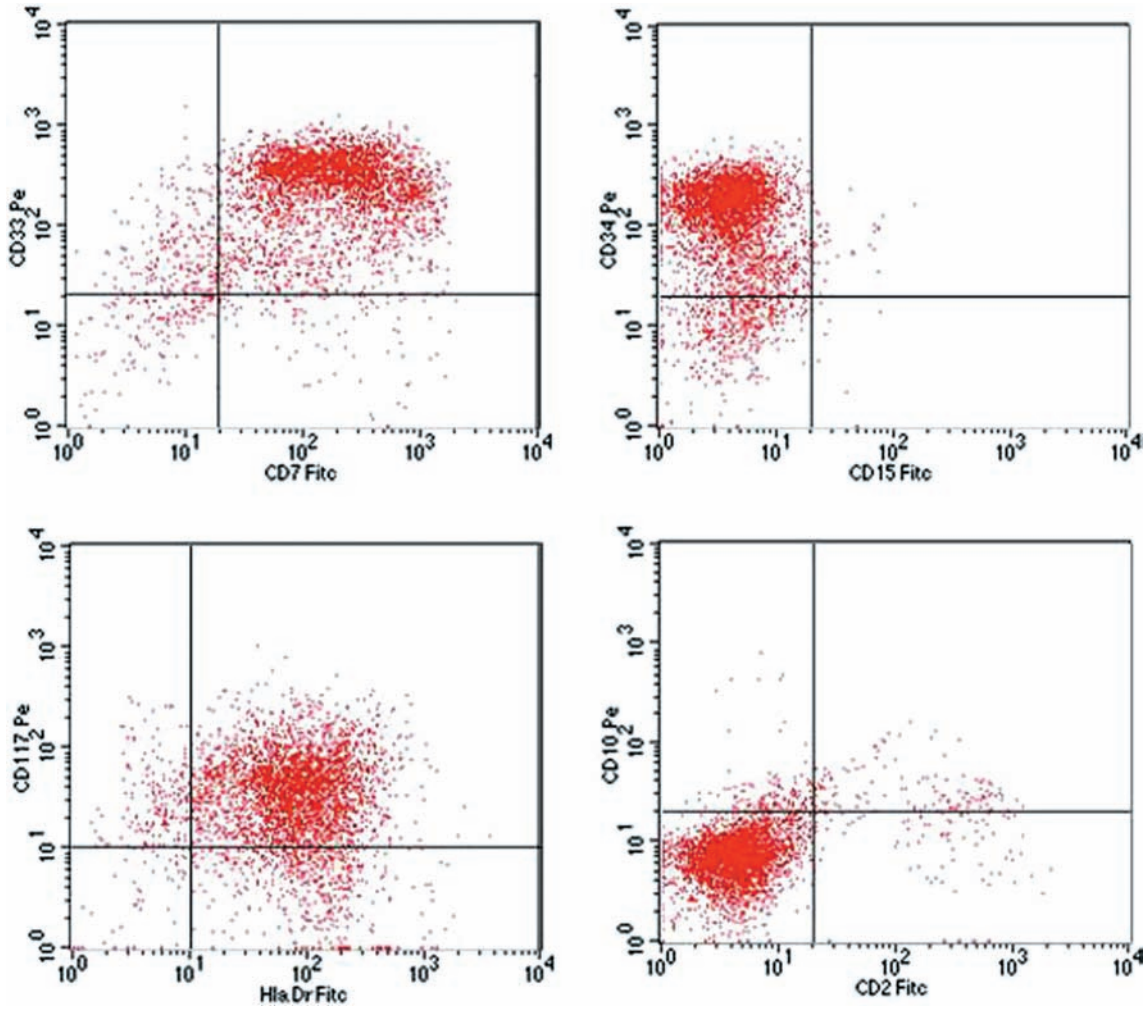


Fig. 2.8 Flow cytometric immunophenotyping in AML showing aberrant antigen expression that could be used for monitoring MRD. There is appropriate expression of CD33 and CD117, which are myeloid-associated antigens, and expression of CD34, a marker of blast cells, but in addition there is aberrant expression of CD7, a T-lymphocyte-associated antigen. (By courtesy of Mr Ricardo Morilla and Professor Daniel Catovsky.)

Immunophenotyping in acute myeloid leukaemia

Immunological markers that identify AML and distinguish it from ALL include reactivity with antibodies of the CD13, CD33, CD65 and CD117 clusters and reactivity with antibodies that recognize the myeloperoxidase (MPO) protein including its proenzyme form. CD117 has a higher degree of specificity for the myeloid lineage than CD13 or CD33 and CD13 is more specific than CD33 [27]. In one study CD13, CD117 and MPO were each expressed in about three quarters of cases of AML while CD33 was expressed in 88% [27]. CD13 is most sensitive when used with a technique that allows cytoplasmic antigen (cCD13) to be detected, since the antigen appears earlier in the cytoplasm than on the cell membrane [28]. The flow cytometric detection of MPO is less sensitive than enzyme cytochemistry if the recommended cut-off points of 10% for the former and 3% for the latter are used, but if 3% is used for both techniques flow cytometry is more sensitive (since the enzymatically inactive proenzyme is also detected); nevertheless there are still a small number of cases that are positive by enzyme cytochemistry and negative by flow cytometry [29].

Table 2.7 Pattern of reactivity with monoclonal (or polyclonal) antibodies commonly observed in French–American–British (FAB) categories of acute myeloid leukaemia (AML). (Derived from references 1, 16 and 30–43, and other sources.)

	Markers of precursor cells			Myeloid markers				Monocyte markers	
	TdT*	HLA-DR†	CD34‡	CD13	CD33	CD117	CD15	CD11b	CD14
M0	Pos. or neg.	Pos.	Pos.	Mainly pos.	Pos. or neg.	Often pos.	Mainly neg.	Mainly neg.	Mainly neg.
M1	Pos. or neg.	Pos.	Mainly pos.	Mainly pos.	Pos.	Often pos.	Mainly neg.	Pos. or neg.	Mainly neg.
M2	Neg.	Pos.	Mainly neg.	Pos.	Pos.	Pos.	Pos.	Pos. or neg.	Mainly neg.
M3§	Neg.	Neg.	Neg.	Pos.	Pos.	Pos. or neg.	Pos. or neg.	Mainly neg.	Mainly neg.
M4	Mainly neg.	Pos.	Pos. or neg.	Mainly pos.	Pos.	Pos. or neg.	Pos.	Pos.	Often pos.
M5	Mainly neg.	Pos.	Pos. or neg.	Pos. or neg.	Pos.	Pos. or neg.	Pos.	Pos.	Often pos.
M6**	Neg.	Pos. or neg.	Pos. or neg.	Pos. or neg.	Pos. or neg.	Pos.	Mainly neg.	Pos. or neg.	Mainly neg.
M7††	Neg.	Mainly pos.	Mainly pos.	Mainly neg.	Pos. or neg.	Often pos.	Mainly neg.	Neg.	Neg.
AML overall	10–20% pos.	About 70% pos.	30–40% pos.	60–90% pos.	70–90% pos.	60–70% pos.	40–70% pos.	50–60% pos.	15–40% pos.

CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase.
* Also positive in acute lymphoblastic leukaemia (ALL).
† Also positive in B-lineage ALL and in occasional cases of T-lineage ALL.
‡ Also positive in many cases of B-lineage ALL.
§ CD9 positive.
** CD36 and CD235a (anti-glycophorin A) positive.
†† CD9, CD36, CD41, CD42a, CD42b, CD61 positive.

The use of a wider panel of McAb shows different patterns of reactivity within the different FAB classes, although the correlation is not very tight [1,16,30–43] (Table 2.7; Fig. 2.9). In addition, light scatter patterns differ between FAB categories. For example, the granular cells of M3 AML have high SSC and this is often also true of M3 variant AML. CD13, CD33, CD65 and anti-MPO antibodies show little difference between the FAB classes while other McAb show some selectivity for immature cells, for more mature cells, for granulocytic differentiation or for monocytic differentiation. CD13 antibodies react with the leukaemic cells of the majority of cases of M1 to M5 AML but with a somewhat lower percentage of cases being positive when there is monocytic differentiation (M4 and M5); CD13 usually also gives positive reactions in M0 AML. CD33 antibodies are somewhat less likely to give positive reactions in M0 AML but reactions are generally positive in M1 to M5 AML. Most CD15 antibodies are generally negative in M0 and M1 AML but are positive in M2, M4 and M5b [31,32,40]. Reactions of CD15 antibodies in AML M3 and M5a are less consistent.

CD11b, CD14 and CD64 antibodies show some specificity for leukaemias with monocytic differentiation. In addition, strong expression of CD45 and high SSC is characteristic of monocytic differentiation. CD14 antibodies are better than CD11b antibodies for distinguishing M4 and M5 AML from M1,

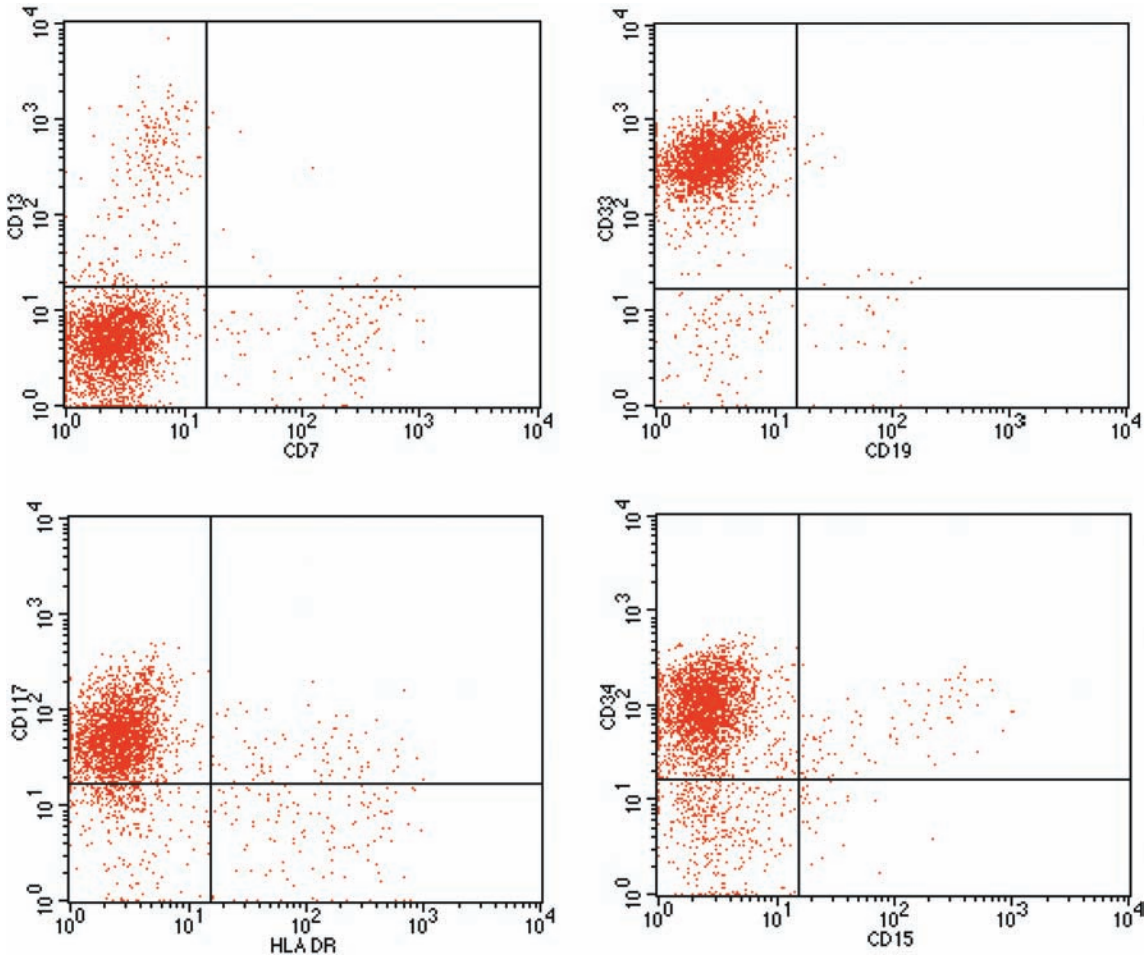


Fig. 2.9 Flow cytometric immunophenotyping in acute promyelocytic leukaemia (FAB M3 AML) showing expression of myeloid-associated antigens, CD33 and CD117, and expression of CD34, a marker of blast cells. There is a failure to express CD13, which is sometimes observed in this subtype of leukaemia, and a very characteristic failure to express HLA-DR. (By courtesy of Mr Ricardo Morilla and Professor Daniel Catovsky.)

M2 and M3 AML [30,32,44]. CD68 is also usually positive in M4 and M5 AML but is only positive in about 40% of other subtypes [36]. Cases of AML that are positive for CD33 and negative for CD13 and CD34 are usually of the M5 subtype [1]. CD116, the receptor for granulocyte–macrophage colony-stimulating factor (GM-CSF), is much more strongly expressed in M5 AML than in other categories [45]. CD87, the urokinase-type plasminogen activator receptor, is also preferentially expressed in M5 AML [46]. M5a and M5b AML show some differences in their pattern of reaction with McAb. The less mature cells of M5a are more likely to give negative reactions with CD13, CD15, CD11b, CD14 [32] and CD68. In comparison with other FAB categories, cases of M0 AML more often express TdT, HLA-DR, CD34 and CD7.

M3 and M3 variant AML show a characteristic pattern of reaction with McAb, which may be of diagnostic importance in distinguishing M3V AML from M5b AML (see Table 2.7 and page 126).

Cases of M4 and M5 AML are usually CD13, CD33, CD4 and HLA-DR positive. CD36 is usually expressed and CD45 expression is strong. Reactions

with CD14, CD16 and CD24 show a high degree of specificity for the monocyte lineage but are not very sensitive. CD64 shows high sensitivity and, if weak reactions in M3 are disregarded, high specificity. CD4 is sensitive but not specific.

Diagnosis of M6 AML, particularly when the cells have an immature phenotype, is aided by the use of immunological markers, but good immunophenotypic markers for very early erythroid cells are lacking. The earliest recognizable erythroid cells express a number of antigens that are not lineage specific including HLA-DR, the transferrin receptor (CD71), certain blood group antigens (A, B and H; I and i) and CD36. Although not specific, CD71 reactivity is suggestive of erythroid differentiation since strong reactivity is rarely present in other myeloid leukaemias [42]. CD36 McAb react also with megakaryoblasts and monocytes [40,47] but can be useful when interpreted in conjunction with other markers. More mature erythroid cells express lineage-specific antigens detectable with either McAb or PcAb. The most commonly employed antibody is anti-glycophorin A (CD235a). Others that have been used include anti-haemoglobin, anti-carbonic anhydrase I [48], anti-spectrin and antibodies to the Gerbich red cell antigen. Carbonic anhydrase I, detectable by a PcAb, is said to be the earliest specific immunophenotypic marker of the erythroid lineage [48].

Immunological markers are important in the diagnosis of M7 AML since they are more specific than cytochemistry and much more widely available than the platelet peroxidase (PPO) reaction, which requires ultrastructural cytochemistry for its detection. The usual order of appearance of markers in the megakaryocyte lineage is probably HLA-DR, PPO and acid phosphatase followed by CD33, CD34 and α-naphthyl acetate esterase activity, followed in turn by platelet glycoprotein IIIa (CD61), glycoprotein IIb and the IIb/IIIa complex (CD41), glycoprotein IX and Ib (CD42a and b) and finally periodic acid–Schiff (PAS) positivity and expression of the von Willebrand antigen. CD41 and CD61 McAb have some advantages over CD42 McAb: they are more sensitive since the antigen appears earlier, and also are more specific since occasional cases of ALL and M5 AML have been found to be positive with CD42 McAb [49]. In the megakaryocyte lineage, only early cells – megakaryoblasts and immature megakaryocytes – show CD33 and CD34 expression. Expression of CD2 and CD7 is common in M7 AML, being observed in 23% and 50% of cases, respectively, in one series [50]. It should be noted that the adhesion of platelets to leukaemic blasts can cause false positivity for platelet antigens in subtypes of AML other than M7. It has therefore been recommended that positive results by flow cytometry be confirmed by immunocytochemistry [1].

The immunophenotype in transient abnormal myelopoiesis of Down syndrome is characteristic [51] (see page 165).

Acute basophilic leukaemia can be identified by expression of either CD123 or CD203c by cells that do not express CD117.

In acute mast cell leukaemia, cells are positive for CD13, CD33, CD117, CD203c and mast cell tryptase (see Fig. 1.55). They also express various antigens not expressed on normal mast cells, specifically CD2, CD25 and CD38 [52].

Neoplastic cells of myeloid leukaemias not infrequently express immunophenotypic markers that are not lineage specific such as TdT, HLA-DR and CD34. TdT is a marker of immature haemopoietic and lymphoid cells. It is positive in the great majority of cases of ALL but in only 15–20% of cases of AML. Expression is stronger in B-lineage ALL than T-lineage ALL and is weaker in AML [53]; among cases of AML, expression is common in FAB categories characterized by a lack of maturation, i.e. in M0 and M1 AML. Expression of TdT correlates with expression of CD7 and CD34 [54]. Expression is most common among cases of M0 and M1 AML and in some series also among cases of M2 and M4 AML [30,35,40,55]. HLA-DR is also expressed on haemopoietic precursor cells but continues to be expressed up to the myeloblast stage in granulocytic maturation and up to the mature monocyte stage in monocyte maturation. It is therefore widely expressed among cases of AML but, as mentioned above, is generally negative in M3 AML. CD133 is expressed in about 40% of patients with AML but it does not distinguish AML from ALL [56]; its expression in AML correlates with other markers of immaturity, being most frequent in M0 AML and not being a feature of M3 AML [56].

Cases of AML may express antigens that are usually viewed as more characteristic of lymphoid leukaemias. The B-lymphoid antigen CD24 is

expressed in the majority of cases of M4 and M5 AML but is rarely expressed in other categories of AML [57]. CD7, which is expressed in T lymphocytes and in many cases of T-ALL, is also expressed in 10–25% of cases of AML with expression being more frequent in M0, M1 and M5 [55]. The T-lymphoid antigen CD4, which is often expressed in M4 and M5 AML, is sometimes expressed in other subtypes. The T-lymphoid antigen CD2 is expressed in a quarter of cases of M3 AML and is occasionally expressed in other subtypes [37,58]. The natural killer cell marker CD56 is expressed in about 20–40% of cases of AML and the natural killer marker CD16 in about a quarter [39,59].

Whether expression of various immunophenotypic markers is of prognostic significance in AML is controversial, with conflicting results having been reported in different series of patients. What prognostic significance has been demonstrated may largely reflect the fact that the immunophenotype provides a surrogate marker of certain cytogenetic abnormalities. Expression of a strongly myeloid phenotype (positivity for MPO, CD13, CD33, CD65 and CD117) has been found to correlate with favourable cytogenetic abnormalities and a better prognosis [60]. Conversely, CD56 expression has been found to correlate with unfavourable cytogenetic abnormalities and with lower complete remission rate and worse survival [61]. CD56 expression has also been associated with a worse prognosis when found in two good prognosis categories of AML, those associated with t(8;21)(q22;q22) and t(15;17)(q22;q12), respectively [62]. Overall, expression of lymphoid antigens in AML has not been found to be of prognostic significance. This is not surprising since expression of a specific lymphoid antigen can be associated with both good and bad prognosis subtypes; for example, CD19 expression is associated with both AML associated with t(8;21) (good prognosis) and with AML associated with t(9;22) (poor prognosis) [62]. CD7 positivity in AML has, however, been found to not only correlate with prognostically worse karyotypic abnormalities but also to be indicative of worse prognosis within the group of patients with the most adverse karyotypes [62,63].

Immunophenotypic techniques can also be adapted to permit detection of proteins that convey multiple drug resistance to AML cells.

Immunophenotyping can be used to assess response to treatment. In one study in AML, the number of cells with a leukaemia-associated immunophenotype at day 16 was found to be of prognostic significance, this being more significant than the percentage of blast cells assessed morphologically [64].

Immunophenotyping is usually performed on suspensions of peripheral blood or bone marrow cells but, when necessary, can be carried out on histological sections, albeit with a more limited range of antibodies. This is most likely to be necessary in M7 AML and in the WHO category of acute panmyelosis when there may be few blast cells in the peripheral blood, and bone marrow fibrosis makes it difficult to obtain an adequate aspirate. Useful antibodies applicable to decalcified trephine biopsy sections are shown in Table 2.8 [65–67]. Immunohistochemistry can identify an acute leukaemia as myeloid and can identify certain FAB categories, e.g. M6 AML and M7 AML. In cases of M5 AML showing the least maturation, leukaemic cells are positive only for lysozyme, showing focal positivity. Cases with more maturation have diffuse lysozyme activity and are also positive with CD68 and Mac387 McAb [68]. However, lysozyme detected by immunohistochemistry does not distinguish between granulocytic and monocytic lineages. Antibodies of the CD68R cluster are more specific for the monocyte lineage than are CD68 McAb.

Immunophenotyping in acute lymphoblastic leukaemia

Immunophenotyping confirms the diagnosis of ALL and separates cases into leukaemias of B lineage and T lineage, which differ in their clinical characteristics. T-ALL may have an associated thymic mass and central nervous system disease at presentation is more likely [69]. T lineage has generally been associated with a higher white blood cell count (WBC) although in one Children's Cancer Group study a WBC above 50×10^9/l was no more common in T-lineage disease than in B-lineage [70]. The haemoglobin concentration (Hb) is more often normal in T-lineage disease, an observation confirmed in the same study [70]. The prognostic significance of lineage differs between different series of patients, indicating that prognosis is determined

Table 2.8 Monoclonal antibodies and polyclonal antisera useful in the diagnosis of acute leukaemia from decalcified paraffin-embedded trephine biopsy specimens [65–67] (for a larger range of antibodies see reference 67).

Category	Antibody	Specificity
CD45	2B11, PD7/26, RP2/18, RP2/22	Leucocyte common antigen: strong reactions in most lymphoid cells (T and B lineage), weak reactions in blasts of myeloid lineage
Anti-TdT	NPT26, SEN28	Positive in lymphoblasts but negative in mature lymphoid cells, positive in blasts in a minority of cases of AML
CD34	QBend/10	Haemopoietic and lymphoid precursors, endothelial cells
CD79a	JCB117, Mb1, HP47/A9	Positive in B-lineage lymphoblasts and lymphocytes
CD10	56C6	Common and pre-B ALL and some Burkitt lymphoma; also positive in follicular lymphoma
CD20	L26	Positive in B-lineage lymphocytes, some B-lineage lymphoblasts and follicular dendritic cells
CD3	CD3-12 and Pc	Positive in T-lineage lymphoblasts and lymphocytes
Anti-MPO	2C7 or Pc	Positive in blasts in AML except in FAB M7 AML and some cases of M0 AML
Anti-neutrophil elastase	NP57	Maturing cells of granulocyte lineage
CD14	Leu M3, NCL-CD14-223	Positive in blasts in some cases of AML, mainly FAB M4 and M5
CD15	Leu M1, BY87	Positive in blasts in some cases of AML and in Reed–Sternberg cells and mononuclear Hodgkin cells
CD68	KP1	Positive in blasts in many cases of AML (also monocytes, macrophages, mast cells and cells of some cases of hairy cell leukaemia and chronic lymphocytic leukaemia); note that KP1 has broad specificity and is thus useful for all FAB classes
CD68R	PGM1	Monocyte restricted
CD117	57A5D8	Haemopoietic progenitors including some proerythroblasts and promyelocytes, blast cells of many cases of AML, mast cells (strongly)
Anti-calprotectin (previously calgranulin)	Mac387	Positive in most FAB M4 and M5 AML; positive with both granulocyte and monocyte lineages
Anti-lysozyme	Anti-lysozyme (Pc)	Positive in granulocyte and monocyte lineages
CD61	Y2/51	Megakaryocytes and blasts of FAB M7 AML
CD42b	MM2/174	Megakaryocytes and blasts of FAB M7 AML
Anti-von Willebrand factor	F8/86 or Pc	Megakaryocytes and blasts of FAB M7 AML
CD235a (glycophorin A)	JC159	Erythroid cells
CD236R (glycophorin C)	ret40f	Erythroid cells

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CD, cluster of differentiation; FAB, French–American–British (leukaemia classification); MPO, myeloperoxidase; Pc, polyclonal; TdT, terminal deoxynucleotidyl transferase.

by an interaction between lineage and treatment given. If Philadelphia (Ph)-positive cases are excluded from the analysis, T-lineage ALL has generally been associated with a worse prognosis; however, in one large series of Ph-negative adult patients T lineage was associated with a better prognosis than B lineage [71]. A higher WBC is indicative of worse prognosis in ALL of either lineage but the count that best separates good prognosis and poor prognosis differs, often being taken as 100×10^9/l in T-lineage disease and 30×10^9/l in B-lineage disease [71]. Although many treatment protocols do not distinguish between T- and B-lineage cases there are some that do, based on the greater sensitivity of T lymphoblasts to asparaginase and their lesser sensitivity to lower doses of methotrexate.

Immunophenotyping is particularly important if the differential diagnosis is between ALL and Burkitt lymphoma, since the latter needs very specific treatment. Immunophenotyping can demonstrate an aberrant lymphoid population in children who present with bone marrow aplasia as a prodrome to ALL [1], can permit the distinction between leukaemic blasts and haematogones (see below), can give prognostic information early in treatment, can indicate targets for monoclonal antibody therapy and can be used for monitoring MRD.

Useful McAb for the identification of B-lineage blasts are CD19, CD79a (cytoplasmic epitope detected) and CD22 (more sensitive when used with a method for detection of cytoplasmic antigen). CD79a is not lineage specific, being expressed in about 10% of T-lineage ALL [72]. CD79b is expressed later in development than CD79a and is thus less useful. CD10 is more strongly expressed in B-lineage blast cells than in T-lineage. CD34 and TdT, when expressed, are useful for excluding a neoplasm of mature B cells. CD34 is expressed in about 70% of cases and TdT in about 97% [27]. It should be noted that, in the WHO classification, cases showing expression of surface membrane immunoglobulin (SmIg) are generally categorized as non-Hodgkin lymphoma whereas the FAB and EGIL classifications categorized such cases as ALL. The WHO approach reflects the fact that immunologically the cells are mature B cells not precursor cells. However, the situation is complex since sometimes lymphoblasts expressing markers of immaturity also show asynchronous expression of SmIg. Detection of SmIg is useful for confirming that cells with L3 cytological features are mature B cells (usually Burkitt lymphoma and sometimes 'blastoid follicular lymphoma') and also contributes to distinguishing blastoid mantle cell lymphoma from ALL. Although the blast cells of B-lineage ALL express B-lineage-associated surface membrane antigens there is evidence that the cell that gives rise to the leukaemic clone is more primitive, expressing CD34 but not CD19 or CD10 [73]. When immunohistochemistry is applied, antibodies to CD79a, CD20, CD22, CD10 and PAX5 are useful.

For T-lineage blasts, the most specific antibody is CD3, which is most sensitive when used with a technique for detection of cytoplasmic antigen (cCD3). Anti-T-cell receptor (anti-TCR) αβ and anti-TCR γδ probably have similar specificity. CD2, CD4, CD5 and CD7 are all less specific. Since CD7 is also expressed in some cases of AML, it is inappropriate to classify a case of acute leukaemia as T-lineage ALL on the basis of reactivity with CD7 alone. Expression of CD1a, CD34 or TdT indicates ALL rather than a neoplasm of mature T cells. Coexpression of CD4 and CD8 or failure to express either also favours a precursor neoplasm. CD56 is expressed in a minority of cases and has been related to a worse prognosis [74]. When immunohistochemistry is used, available antibodies include those directed at CD1a, CD2, CD3, CD4, CD5, CD7 and CD8.

It should be noted that a third or more of cases of ALL fail to express CD45, which is expressed on all normal T and B lymphocytes [75]. This must be remembered if a gating protocol uses CD45 since gating on a blast window defined by CD45 expression and SSC may mean that blast cells are missed.

The use of wider panels of antibodies permits the further separation of T-lineage and B-lineage ALL into categories that are believed to reflect the normal maturation within these lineages. More importantly, in the case of B-lineage ALL, these categories show some correlation with cytogenetic subsets of ALL and consequently indicate differences in prognosis. A number of classifications and terminologies have been proposed, that of the EGIL group being shown in Tables 2.9 [13] and 2.10 [15]. It should be noted that HLA-DR is expressed in the great majority of cases of B-lineage ALL, regardless of the maturity of the cell, whereas, among cases of T-lineage ALL, HLA-DR expression correlates with an immature immunophenotype. Among B-lineage cases, common and pre-B ALL have a similar prognosis whereas the prognosis of early-B ALL is worse, even if the poor-risk group of infants less than a year of age are excluded [76]. A small subset of patients within the pre-B ALL group are found to have CD10-negative cells that often show expression of myeloid antigens and reactivity with antibody 7.1; these cases may be associated with t(4;11) or *MLL* rearrangement and have a poor prognosis whether or not *MLL* is rearranged [77]. Among T-lineage cases, the precise immunophenotype appears to be of less significance.

Classifications of B-lineage ALL reflect a putative normal sequence of B-cell maturation in which

Table 2.9 European Group for the Immunological Characterization of Leukemias (EGIL) classification of B-lineage acute lymphoblastic leukaemia [15].

All categories are positive for CD19 and/or CD79a and/or CD22; most cases, except mature B, are TdT positive	
B-I (pro-B)	CD10–, anti-cμ–, anti-SmIg–
B-II (common)	CD10+, anti-SmIg–, anti-cμ–
B-III (pre-B)	Anti-cμ+
B-IV (mature-B)*	Anti-c or Sm κ or λ

c, cytoplasmic; CD, cluster of differentiation; Ig, immunoglobulin; Sm, surface membrane; TdT, terminal deoxynucleotidyl transferase.
* Now regarded as non-Hodgkin lymphoma rather than acute lymphoblastic leukaemia.

Table 2.10 European Group for the Immunological Characterization of Leukemias (EGIL) classification of T-lineage acute lymphoblastic leukaemia [15].

All cases are positive for c or Sm CD3; some cases are CD10 positive	
T-I (pro-T)	CD7+, CD2–, CD5–, CD8–, CD1a–
T-II (pre-T)	CD2+ and/or CD5+ and/or CD8+, CD1a–
T-III (cortical-T)	CD1a+, membrane CD3+ or –
T-IV (mature-T)	Membrane CD3+, CD1a–
Group a	Anti-TCRαβ+
Group b	Anti-TCRγδ+

c, cytoplasmic; CD, cluster of differentiation; Sm, surface membrane; TCR, T-cell receptor.

early cells express only HLA-DR, TdT and pan-B antigens such as CD19, cCD22 and CD79a. Subsequently there is expression of CD24 and CD10 followed by the appearance of cytoplasmic μ chain (cμ) and CD79b, then cytoplasmic κ and λ chain and, finally, SmIg. CD34 is usually expressed in pro-B and common ALL but not pre-B or mature-B ALL [1]. TdT is usually positive in pro-B and common ALL but may be negative in pre-B ALL [1]. Overall, TdT is negative in approaching 3% of B-lineage ALL [78]. Coexpression of myeloid antigens, CD13 and CD33, is more common in early B precursor (pro-B) ALL but has not been found to be of any prognostic significance [76]. It should be noted that the category 'common ALL' does not necessarily include all cases expressing the common ALL antigen (CD10) since expression may be seen in pre-B ALL and sometimes in Burkitt lymphoma. Cases with expression of both CD10 and SmIg are generally not ALL, rather representing non-Hodgkin lymphoma. Expression of cytoplasmic κ or λ chain also suggests non-Hodgkin lymphoma rather than ALL. It should be noted that the use of the designation 'B-ALL' for cases expressing SmIg is ambiguous and can lead to confusion. Despite the previous use of this term to indicate a mature B phenotype, the 2008 WHO classification of lymphoid neoplasms suggests its use to indicate B-lineage ALL. This seems unfortunate. The recognition of early precursor or pro-B ALL may be important since the prognosis is generally worse than that of common ALL. The identification of pre-B cases was at one stage considered important since such cases included a cytogenetic subgroup, t(1;19)(q23;p13), which was previously associated with an unfavourable prognosis; since the prognosis of this subtype is greatly improved with current treatment, identification of pre-B cases that may have t(1;19) is no longer important for determining prognosis and choice of treatment. It has been suggested that a category of transitional pre-B ALL in which there is expression of surface and cytoplasmic μ chains without expression of κ or λ light chains should be distinguished from other pre-B ALL [79]. This subtype, which is not associated with any specific karyotypic abnormality, has a good prognosis with standard therapy. CD20 expression has been associated with a worse prognosis in adults; in children no such adverse effect is seen. The adverse prognosis in adults is converted into a better prognosis if rituximab is included in the treatment [80]. High expression of CD40 has been found to correlate with better relapse-free survival in B-lineage ALL on multivariate analysis [81]. As cytogenetic and molecular genetic investigation of cases of ALL has become more widespread the importance of immunophenotyping in identifying unfavourable prognostic categories of B-lineage ALL and thus influencing treatment has become much less important.

Immunophenotyping has a role in distinguishing precursor-B leukaemic lymphoblasts from immature normal or reactive cells, known as haematogones. A proportion of haematogones express markers of immaturity such as CD34, TdT, CD10

and CD43 [82]. However, they differ from leukaemic lymphoblasts in that the population of cells ranges from immature to mature, in contrast to the more consistently immature and often aberrant immunophenotype of leukaemic lymphoblasts [83]. Recognition of these differences is best achieved with multicolour immunophenotyping since merely measuring the percentage of cells expressing different antigens may be misleading. Scatter plots show that leukaemic lymphoblasts form a much more compact cluster than haematogones, which show a spectrum of antigen expression, ranging from cells expressing CD34, CD43, CD10 and TdT to those expressing none of these markers but expressing CD20. BCL2 expression on cells falling within the blast window on CD45/light-scattering characteristics has also been recommended to aid in the distinction between residual B-lineage blast cells and haematogones [84]. The differences between haematogones and leukaemic lymphoblasts are summarized in Table 2.11.

Immunophenotyping of B-lineage cases early in treatment can give major prognostic information depending on the number of residual cells expressing CD19 and either CD10, CD34 or both [85]. Normal cells with this phenotype are very sensitive to corticosteroids so that residual cells with this phenotype are likely to be leukaemic cells; if leukaemic cells are not sensitive to corticosteroids the prognosis is much worse.

Classifications of T-lineage ALL essentially divide cases into two groups with immunophenotypes analogous to those of early and cortical (or common) thymocytes, respectively, and a third group analogous to mature thymocytes or to T cells. In some classifications the first two categories are amalgamated [11] and in others the categories are increased to four, as in the EGIL classification [15] (see Table 2.10). HLA-DR and TdT expression are less likely with the more mature immunophenotypes. Overall, about 95% of cases express TdT but HLA-DR and CD34 are usually not expressed [27]. The most immature phenotype may show expression of CD13, CD33 and CD56 [86]. The various categories of T-lineage ALL have been found to show some prognostic differences but these are less marked than in the case of B-lineage ALL. In one study, using a classification proposed by the Pediatric Oncology Group, children whose lymphoblasts had an early thymocyte phenotype had an appreciably lower remission rate than those whose lymphoblasts had an intermediate or late phenotype, but there was no difference in event-free survival [87]. In another childhood study, using the same classification, CD3 positivity and CD10 negativity were associated with a worse prognosis but only CD10 negativity was an independent prognostic variable [88]. However, in a study in adults CD10 was not of prognostic significance [89]. In a German multicentre study in adults, cases classified as pre-T

Table 2.11 A comparison of the immunophenotypic characteristics of haematogones and leukaemic lymphoblasts of B lineage.

Haematogones	Leukaemic lymphoblasts
Spectrum of cells from immature to mature (e.g. variable TdT and CD20)	Cells apparently arrested at one stage of maturation
Overall, TdT and CD38 more strongly expressed	Overall, TdT and CD38 more weakly expressed
Overall CD10, CD19 and CD58 more weakly expressed	Overall CD10, CD19 and CD58 more strongly expressed
Surface membrane antigens expressed synchronously and with strength of expression appropriate to stage of maturation	Surface membrane antigens expressed asynchronously (e.g. coexpression of CD34 and CD20, coexpression of CD10 and strong CD22) or inappropriately weakly or strongly (e.g. absent or weak CD45, absent CD20, absent CD22, weak CD38, weak or absent CD10, weak CD19)
No aberrant antigen expression	Frequent aberrant expression of myeloid antigens (most often CD13, CD15, CD33) or CD7

CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase.

(E-rosette-forming cells (ERFC) negative) had a worse prognosis than T-cell cases (ERFC positive) [90]. In a further study of adults and children, those with an 'early' phenotype (SmCD3– CD1–) were more likely to be adults and although they had a lower mean WBC the prognosis for survival was worse than in other cases [91]. Several studies have shown CD1a expression to be associated with a better prognosis than either a more mature or a less mature immunophenotype [62]. In an Italian study of adults, those with a pro-T or pre-T immunophenotype had a significantly lower rate of complete remission than those with a cortical thymocyte or mature-T immunophenotype [89]. Several studies have expression of TCR$\gamma\delta$ to be associated with a better prognosis than expression of TCR$\alpha\beta$ [62]. Although there is a consensus that the immunophenotype in T-lineage ALL correlates with disease characteristics and, to some extent, with prognosis, this is not generally regarded as an indication for an alteration of management. Further categorization of T-lineage cases is thus of less importance than further categorization of B-lineage cases. In T-lineage cases, in contrast to B-lineage, there is little relationship between immunophenotype and specific chromosomal abnormalities, although a higher frequency of normal karyotype in cases with an immature phenotype has been reported [92].

Expression of myeloid antigens is not generally of prognostic significance in ALL [62], although CD15 and CD65 expression in pro-B ALL may point to adverse disease associated with t(4;11). In one study of adult T-lineage ALL, a lower rate of complete remission correlated with expression of CD13, CD33 and CD34 but in multivariate analysis only correlation with CD33 remained significant [89]. A large study in adults with B- or T-lineage ALL by the same group found expression of CD13, CD33 or both to be lacking in prognostic significance [93].

If immunophenotyping is to be used for the detection of MRD in ALL, it is necessary to use an appropriate panel of antibodies to recognize a leukaemia-associated immunophenotype [94]. Characteristics sought are either asynchronous expression of antigens (either coexpression of markers that are normally expressed on mature and immature cells respectively or failure to express a marker that is usually expressed at the same stage of maturation as another marker), aberrant expression of antigens, inappropriately weak or strong expression of antigens, or expression of a marker or combination of markers on bone marrow or blood lymphoid cells that is normally expressed only by thymic cells. Expression of aberrant myeloid markers is common in T-lineage ALL, both at diagnosis and at relapse [95]. Such expression is uncommon at presentation of B-lineage ALL but at relapse it is significantly more common [95]. In general, at least three antigens need to be studied simultaneously for effective detection of MRD. Some of the range of abnormalities that have been used are shown in Table 2.12. Detection of MRD by flow cytometry is less sensitive in B-lineage ALL than T-lineage ALL because of possible confusion of leukaemic blast cells with increased normal B-cell precursors.

Immunophenotyping can also produce information of use in planning therapy with monoclonal antibodies. CD20 is of potential value in cases of ALL that express this antigen [96]. Similarly, CD33 antibodies may be useful in cases showing aberrant expression of this antigen [96].

In addition to assigning lineage and identifying aberrant antigen expression, flow cytometry can be used for quantitating the amount of DNA in leukaemic cells using a fluorochrome that binds stochiometrically to DNA. The DNA index compares the amount of DNA with the amount in the nuclei of normal gender-matched cells, e.g. lymphocytes. A DNA index of 1.16 or more indicates a modal chromosome number of 54 or more and correlates with a better survival; it can be used as one of the criteria to assign a child with ALL to a good risk group [26]. Quantitating DNA can also detect multiploidy, i.e. the presence of clones that differ in their number of chromosomes. The detection of a small clone of severely hypodiploid cells is likely to indicate an adverse prognosis.

Immunophenotyping in mixed phenotype acute leukaemia

Cases of acute leukaemia previously designated either biphenotypic or bilineage are recognized; acute biphenotypic leukaemia is one in which leukaemic cells simultaneously expresses markers of two lineages, usually lymphoid and myeloid, whereas acute bilineage or bilineal leukaemia is one in which the leukaemic cells differentiate to distinct

Table 2.12 Typical antibody combinations for the identification of a leukaemia-related immunophenotype that can be used for detection of minimal residual disease.

Antibody combination	Abnormality detected
B-lineage ALL	
TdT plus CD10 coexpressed with CD13, CD15, CD33, CD65, CD66c or 7.1/NG.2	Aberrant expression of a myeloid antigen
CD34 plus CD19 coexpressed with CD13, CD15, CD33, CD65, CD66c or 7.1/NG.2	Aberrant expression of a myeloid antigen
CD19 plus CD10 coexpressed with CD13, CD15, CD33, CD65, CD66c or 7.1/NG.2*	Aberrant expression of a myeloid antigen
TdT plus CD10 coexpressed with CD56 *or* CD34 plus CD19 coexpressed with CD56	Aberrant expression of a natural killer/myeloid antigen
TdT plus CD10 coexpressed with strong CD19, CD21 or CD22	Asynchronous expression
CD34 plus CD10 coexpressed with strong CD19, CD21 or CD22	Asynchronous expression
CD34 plus CD10 plus CD19 coexpressed with strong CD58	Asynchronous expression
TdT plus CD34 coexpressed with μ	Asynchronous expression
T-lineage ALL	
TdT or CD34 on peripheral blood or bone marrow CD3+ T cells	Normally expressed only on thymic cells
CD1a	Normally expressed only on thymocytes, not on peripheral blood or bone marrow cells
CD4 coexpressed with CD8	Normally only coexpressed on thymic cells
AML	
CD33 overexpressed	Stronger expression than on normal cells
CD34 plus TdT plus CD65	Asynchronous expression
CD34 coexpressed with CD11b, CD14, CD15, strong CD33 or CD56	Asynchronous expression
CD117 plus CD11b or CD15	Asynchronous expression
CD13+ CD33–	Lack of synchronous expression
CD13– CD33+	Lack of synchronous expression
CD13– CD15+	Lack of synchronous expression
HLA-DR+ CD15+	Asynchronous expression
CD34+ CD33+ HLA-DR–	Lack of synchronous expression
CD34+ CD117+ HLA-DR–	Lack of synchronous expression
CD117+ CD33+ HLA-DR–	Lack of synchronous expression
Coexpression of myeloid markers with CD2, CD3, CD5 or CD7	Aberrant expression of a T-lymphoid marker
Coexpression of myeloid markers with CD19 or CD20	Aberrant expression of a B-lymphoid marker
Expression of myeloid markers on blasts showing light scatter characteristics more typical of lymphoid cells or vice versa	Expression of markers on cells showing an inappropriate light scatter pattern

c, cytoplasmic; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CD, cluster of differentiation; TdT, terminal deoxynucleotidyl transferase.
* 7.1/NG2 is a monoclonal antibody recognizing chondroitin sulphate.

populations of cells of different lineages. The distinction is to some extent artificial. Criteria for recognition of acute biphenotypic leukaemia were proposed by Mirro and Kitchingman [97] and by the EGIL group [15]. The latter criteria were accepted in modified form in the 2001 WHO classification of tumours of haematopoietic and lymphoid tissues for cases designated 'acute leukaemia of ambiguous lineage' [98]. In the 2008 WHO classification different criteria are suggested for what is now designated mixed phenotype acute leukaemia [99]. These new criteria are summarized in Table 3.9 together with criteria for undifferentiated acute leukaemia. Specific criteria are needed for these categorizations because aberrant antigen expression is common in acute leukaemia and expression of a single aberrant marker, or possibly more than one aberrant marker, does not necessarily

indicate that a leukaemia differs in nature from similar cases without aberrant antigen expression.

Immunophenotyping in suspected B-lineage lymphoproliferative disorders

Immunophenotyping in suspected B-lineage lymphoproliferative disorders can be done on the peripheral blood (if suspect cells are present) or on bone marrow or lymph node cells. The first question to be answered is whether there are increased numbers of B cells or of B cells with an unusual phenotype. The next question to consider is whether a population of B cells is reactive or neoplastic. If cells show light chain restriction (i.e. they express either κ or λ but not both) they are usually neoplastic, although this is not invariably true. If they fail to express SmIg then a uniform immunophenotype suggests a neoplastic clone, particularly if there is aberrant expression of unexpected antigens. If, after immunophenotyping, it remains uncertain whether apparently abnormal cells are clonal and neoplastic, recourse can be had to molecular genetic analysis for the detection of immunoglobulin heavy chain gene (*IGH*) rearrangement. Detection of an abnormal population should be followed by assessment of whether the cells are mature or immature. This may be readily apparent from the blood film but sometimes, for example in blastoid mantle cell lymphoma, cells that appear immature on cytology may be immunophenotypically mature. Mature cells will not express CD34 or TdT. They often express FMC7 and usually express SmIg (although some neoplastic B cells fail to express SmIg or express it only weakly). They express CD45 more strongly than blast cells and their light-scattering properties help to distinguish them from blast cells. Finally, immunophenotyping can be used to support a specific diagnosis, to identify prognostic markers, to identify an aberrant phenotype that could be used for monitoring MRD after treatment, and to identify expression of antigens that could be targets for McAb treatment.

Immunophenotyping in lymphoproliferative disorders of mature B cells will usually readily answer the question as to whether a patient has CLL or something else and as CLL is by far the most common of the lymphoproliferative disorders it is important that the initial antibody panel is directed at its recognition. If the disorder does not appear to be CLL on immunophenotyping and the initial antibody panel was not extensive, further antibodies should be applied. If clinical features and microscopy suggest that another diagnosis is likely, the initial panel can be directed at this possibility. For example, suspected hairy cell leukaemia would lead to application of a specific panel.

In some cases there is a considerable admixture of clonal B cells with non-clonal B cells and T cells. This necessitates gating on B cells, preferably on B cells with an abnormal immunophenotype, for further analysis. It is meaningless, for example, to know the percentage of lymphocytes that express CD5; what is diagnostically important is whether there are B cells expressing CD5. Gating on CD19-positive cells will permit this question to be answered. Assessment of the κ : λ ratio is also more accurate if done on gated (e.g. CD19-positive) B cells, particularly when B cells are a low proportion of total cells.

It is necessary to assess the strength of expression of antigens in relation to an isotype control or a population of cells not expressing the antigen or, in the case of immunoglobulin light chains, by relating the strength of expression of the chain that is expressed to that which is not expressed. The latter technique necessitates using the same fluorochrome and standardizing the procedure so that on normal B cells κ and λ are similarly expressed. Other advantages are derived from the converse approach of combining anti-κ and anti-λ antibodies labelled with different fluorochromes in a single tube. This permits the detection of false-positive results as the result of binding of immunoglobulin to Fc receptors, which will lead to apparent expression of κ and λ on the same cells. It can also be diagnostically useful to compare the strength of CD19 to that of CD20 since detection of down-regulation of CD20 expression is of use in diagnosis.

Details of the interpretation of immunophenotyping in individual lymphoproliferative disorders are discussed in Chapter 6.

Immunophenotyping in suspected T-lineage lymphoproliferative disorders

The purpose of immunophenotyping in suspected T-lineage lymphoproliferative disorders is fourfold: (i) to provide evidence that suggests clonality and thus supports the diagnosis of leukaemia/lymphoma;

(ii) to distinguish precursor T cells from mature T cells; (iii) to provide evidence of a specific subtype of leukaemia/lymphoma; and (iv) to identify expression of antigens, such as CD52 or CD25, that could be relevant to monoclonal antibody treatment.

A T-lineage lymphoproliferative disorder may be suspected from clinical and cytological features or because the initial panel of antibodies, directed at identifying CLL, showed an excess of T cells and no evidence of B-lineage disease. A T-cell panel is then appropriate. There is no easy way to demonstrate clonality of T cells. However, clonality can usually be inferred when there is a population of cells with a uniform but abnormal immunophenotype. The aberrancy of the phenotype may be: (i) weak or absent expression of pan-T antigens, such as CD7; (ii) unusually strong expression of pan-T antigens; or (iii) aberrant expression of myeloid or B-lineage antigens such as CD13, CD15, CD33 or, rarely, CD20. In addition to inferring clonality from a uniform aberrant phenotype, a range of antibodies to different T-cell receptor β chain variable regions are now available and their use can identify presumptive clonality (possible for 65–75% of mature T-cell neoplasms) [100]; the evidence of clonality may be either expression of a single variable region family or failure to express any variable region family despite being TCRαβ positive. Another new technique is the use of antibodies of the CD158 (killer inhibitory receptor) clusters, which can provide evidence of clonality when restricted or absent expression of CD158 epitopes is shown. A very abnormal CD4 : CD8 ratio is seen in clonal disorders but also sometimes when T cells are reactive; for example, B-cell lymphoma can be associated with a marked increase in CD8-positive T cells. It should also be noted that aberrant phenotypes can be seen in reactive conditions (e.g. CD8+ CD7 weak in infectious mononucleosis) [6] and in certain congenital immune deficiencies (CD4– CD8–).

As for the B lineage, expression of CD34 or TdT excludes a diagnosis of T non-Hodgkin lymphoma (except of course precursor-T lymphoblastic lymphoma, which is a variant of ALL). Expression of CD1a also indicates a precursor-T cell. Coexpression of CD4 and CD8 is less specific for a precursor-T cell since aberrant coexpression is sometimes seen in T non-Hodgkin lymphoma or leukaemia (e.g. in T prolymphocytic leukaemia). CD45 expression is usually stronger in mature T cells than in T-lineage blast cells but sometimes CD45 expression is down-regulated or lost so that on CD45/SSC plots the cluster resembles a cluster of blast cells.

The use of immunophenotyping to aid in the differentiation of individual subtypes of T-lineage lymphoproliferative disorders is discussed in more detail in Chapter 6.

Immunophenotyping in suspected NK-lineage lymphoproliferative disorders

NK (natural killer) cells usually express CD2, CD7, CD16 and CD56 and show variable expression of CD8 and CD57; they do not express CD5. NK cells express CD3ε (detected by polyclonal antisera and some monoclonal antibodies used for immunohistochemistry) and activated NK cells express CD3ζ but NK cells do not express the complete CD3 receptor complex. It should be noted that the antigens most typical of the NK lineage lack specificity for this lineage. CD2 and CD7 are expressed by T cells, CD7 by some AML blast cells and CD56 by the neoplastic cells of blastic plasmacytoid dendritic cell neoplasm and some cases of AML, multiple myeloma and small cell carcinoma of the lung. It is thus always necessary to use a panel of antibodies.

Demonstration of presumptive clonality is difficult for NK cells but may be achieved by showing restricted or absent expression of CD158 epitopes.

Genetic analysis

Genetic analysis is of increasing importance in haematological neoplasms, being relevant to: (i) identification of aetiological factors; (ii) diagnosis and classification; (iii) determination of prognosis; and (iv) identification of MRD. Techniques include conventional cytogenetic analysis, molecular genetic analysis of DNA, molecular genetic analysis of ribonucleic acid (RNA), and immunological techniques that depend on recognition of a protein encoded by a specific gene.

Cytogenetic analysis

The value of cytogenetic analysis in haematological neoplasms relates to the fact that the neoplastic clone often has an identifiable acquired cytogenetic abnormality. In the bone marrow or other infiltrated

tissue the karyotypically abnormal cells displace normal cells. Cytogenetic analysis is conventionally carried out by microscopic analysis of the chromosomes of cells arrested in metaphase. The bone marrow or other cells may be examined directly or after a period in culture with or without various mitogens and synchronizing agents. All of a population of leukaemic cells may show the same chromosomal abnormality or further clonal evolution may have occurred so that there are cells with an additional abnormality (or more than one additional abnormality), which represent a daughter clone or subclone (in cytogenetic terminology a sideline derived from the stemline). Before examination the chromosomes are stained with a Giemsa stain or with quinacrine to establish a banding pattern characteristic of each chromosome (Fig. 2.10). Stained chromosomes are numbered in relation to their size and the position of the centromere. The chromosome arms are divided into numbered regions, bands and sub-bands, outwards from the centromere, so that they can be easily described. Various terms and abbreviations used in describing chromosomes and their abnormalities are shown in Table 2.13 [101]. Translocations may be reciprocal (material being exchanged between chromosomes) or non-reciprocal (material from one chromosome being transferred to another). A balanced translocation is one in which there is no net gain or loss of chromosomal material, whereas an unbalanced translocation is one in which translocation is associated with loss or duplication of all or part of a chromosome. Chromosomal abnormalities are described according to the International System for Human Cytogenetic Nomenclature [101]. Translocations are written as follows: t(15;17)(q22;q12), which indicates that there is a reciprocal translocation between chromosomes 15 and 17; the breakpoints are at band q22 on chromosome 15 and at band q12 on chromosome 17. This is the translocation found in acute promyelocytic leukaemia, the first specific abnormality to be linked to a morphologically recognizable subtype of acute leukaemia. In describing translocations, the chromosomes are listed in numerical order. In describing insertions, the chromosome into which material is inserted is listed first followed by the chromosome from which material has been derived. Semicolons must be used in a precise manner in cytogenetic notation: they are used to separate breakpoints on different chromosomes but not to separate the breakpoint of an inversion. Thus we have t(16;16)(p13.1;q22) but inv(16)(p13.1q22). Similarly, the breakpoints at either end of a segment of a chromosome that is inserted into another are not separated by a semicolon. Thus we have ins(5;11)(q31;q13q23) indicating that a part of chromosome 11 extending from band q13 to band q23 has been inserted into chromosome 5 at band q31.

Problems and pitfalls in cytogenetic analysis

It is necessary to distinguish constitutional from acquired abnormalities before using a cytogenetic abnormality as evidence of clonality or neoplasia. Some acquired abnormalities do not indicate a neoplastic condition, e.g. loss of the Y chromosome

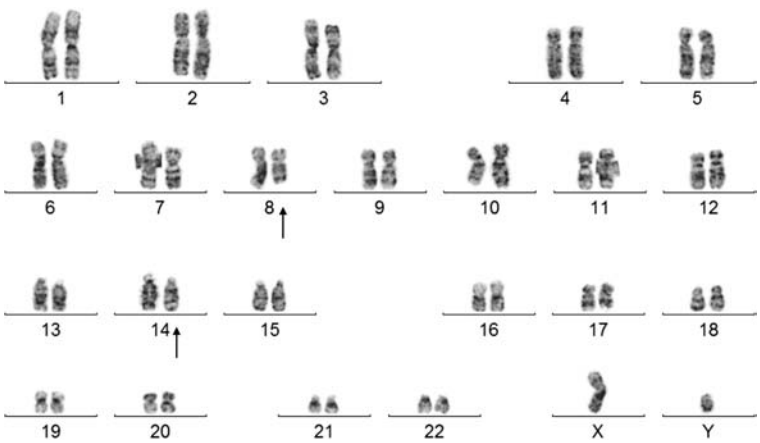


Fig. 2.10 Karyogram from a patient with Burkitt lymphoma showing chromosomes identified by Giemsa banding. There is t(8;14)(q24;q32) with the part of chromosome 8 that is translocated being longer than the part of 14 that is reciprocally translocated; as a result there is shortening of the long arm of chromosome 8 (arrow) and lengthening of the long arm of chromosome 14 (arrow). An extra band derived from chromosome 8 is apparent at the end of chromosome 14. (By courtesy of Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

Table 2.13 Abbreviations, terminology and symbols used in describing chromosomes and their abnormalities.

add	Additional material of unknown origin
aneuploid	Cells having an abnormal number of chromosome that is neither half nor a multiple of 46
band	A transverse light- or dark-staining area of a chromosome, a subdivision of a region, numbered outwards from the centromere
c	Constitutional anomaly, appears after the anomaly, e.g. 47,XX,+21c in Down syndrome
cen	Centromere, the junction of the short arm (p) and the long arm (q), in a karyotype indicated, if necessary, as 10
clone	A cell population from a single progenitor; presumptive evidence is the presence of at least two cells with the same aberration or at least three cells with loss of the same chromosome
cp	Composite karyotype
del	Deletion, may be interstitial or terminal
der	Derivative chromosome, an abnormal chromosome derived from two chromosomes or from more than one rearrangement within a chromosome; it takes its number from the chromosome that contributes the centromere
dic	Dicentric, a chromosome with two centromeres
diploid	Cells having the normal complement of 46 chromosomes (23 pairs); near diploid 35–57 chromosomes
dmin	Double minute (*see* min)
dup	Duplication, extra copy of a segment of a chromosome
haploid	Cells with 23 (unpaired) chromosomes; near haploid = <23–34 chromosomes
high hyperdiploid	Having more than 50 chromosomes
hyperdiploid	Having more than 46 chromosomes
hypodiploid	Having fewer than 46 chromosomes, usually 35–45
hsr	Homogeneously staining region, indicative of amplification (multiple copies) of a small segment of a chromosome
i	Isochromosome, a chromosome formed by duplication of the long arm or the short arm
idem	Latin, the same; denotes the stemline karyotype in a subclone, e.g. 46,XY,t(9;22)(q34;q11.2)[5]/45, idem,-7[3]
idic	Isodicentric chromosome (duplication of either the long arm or the short arm with two centromeres)
ins	Insertion, movement of a segment of a chromosome to a new position on the same or another chromosome; may be direct (dir) or inverted (inv); in the karyotype of an insertion the chromosome into which the insertion is made is listed first
inv	Inversion, i.e. rotation and rejoining of a segment of a chromosome
ish	*In situ* hybridization
karyogram	Systematized array, usually a photograph or a digitized image, of the chromosomes of a cell and by extension of a clone of cells (or an individual); chromosomes are displayed in decreasing order of size, which corresponds to increasing chromosome number; the sex chromosomes, X and Y, are displayed last
karyotype	Written description of the chromosomal make up of a cell and by extension of a clone of cells (or an individual)
mar	Marker chromosome, an abnormal chromosome that cannot be characterized and is therefore of unknown origin
min	Minute, an acentric fragment smaller than the width of a single chromatid; may be single or double (dmin)
ml	Main line: the most frequent chromosomal constitution, not necessarily the initial one
monosomy	Loss of an entire chromosome so that there is only a single copy, indicated by a minus sign (–) before the chromosome number, e.g. –7
p	Short arm of a chromosome (from *petit*)
p+	Lengthening of the short arm of a chromosome
p–	Shortening of the short arm of a chromosome
paracentric inversion	Inversion of a segment of a chromosome confined to one arm
pericentric inversion	Inversion of a segment of a chromosome composed of part of both arms and the centromere

Continued

Table 2.13 (*Continued*)

pseudodiploid	Cells having 46 chromosomes but with structural abnormalities being present
q	Long arm of a chromosome
q+	Lengthening of the long arm of a chromosome
q–	Shortening of the long arm of a chromosome
r	Ring chromosome, derived from one or more chromosomes
region	One of the primary divisions of a long or short arm of a chromosome, numbered outwards from the centromere, further divided into bands
sdl	Sideline: a subclone derived from the stemline
sl	Stemline: the most basic clone of a neoplastic population
sub-band	A transverse light- or dark-staining area within a band of a chromosome, revealed by high resolution techniques, numbered outwards from the centromere; 9q34.3 indicates region 3, band 4 and sub-band 3 of the long arm of chromosome 9 (this is the site of the *ABL1* gene)
t	Translocation, movement of a segment of one chromosome to form part of another chromosome; a translocation is often reciprocal; a translocation may be described as balanced (no loss of chromosomal material detected on microscopic examination of metaphase spreads) or unbalanced (a segment of chromosome is seen to have been lost)
ter	Terminal or telomere
tetraploid	Cells having 92 chromosomes (four sets); near tetraploid = 81–103 chromosomes
triploid	Cells having 69 chromosomes (three sets); near triploid = 58–80 chromosomes
trisomy	Three copies of a chromosome, indicated by a '+' before the chromosome number, e.g. +8
( )	Parentheses, surround chromosomes or breakpoints
[ ]	Square brackets, surrounds the number of cells
,	Comma, separates number of chromosomes, sex chromosomes and chromosome abnormalities, e.g. 46,XY,inv(3)(q21q26.2)
.	Decimal point, separates band number from sub-band number
;	Semicolon, separates chromosomes and breakpoints in structural rearrangements
+	Addition of a chromosome, whether a chromosome is additional being assessed in relation to the ploidy of the cell; also used (but not in a karyotype) to indicate lengthening of one arm of a chromosome, used to indicate gain of a sex chromosome but not a constitutional supernumerary chromosome
–	Loss of a chromosome, whether a chromosome is lost being assessed in relation to the ploidy of the cell; also used (but not in a karyotype) to indicate shortening of one arm of a chromosome, e.g. 5q–; used to indicate loss of a sex chromosome but not constitutional absence
?	Question mark, uncertain identification
———	Single underlining, can be used to identify one of two homologous chromosomes
~	Used when there is uncertainty as to a breakpoint, indicates the segment in which it occurs
/	Separates clones
//	Separates chimaeric clones, e.g. a post-transplant karyotype 46,XX,t(9;22)(q34;q11.2)[6]//46,XY[14] indicates six recipient metaphases in a patient with chronic granulocytic leukaemia and 14 donor metaphases

is more often an age-related phenomenon. Clonal abnormalities sometimes occur without any apparent haematological disorder, e.g. being observed, rarely, in donor cells following haemopoietic stem cell transplantation [102]. Conversely, cytogenetic abnormalities may be absent in neoplastic conditions. Sometimes no mitoses are obtained or only normal cells enter mitosis, even though neoplastic cells with a clonal abnormality are present. Some chromosomal rearrangements are difficult to detect, for example because the parts of chromosomes that are reciprocally exchanged are similar in their size and banding pattern, e.g. t(12;21)(p13;q22) in ALL. Other rearrangements, e.g. the small interstitial deletion leading to a *FIP1L1-PDGFRA* fusion gene in chronic eosinophilic leukaemia, are below the level of resolution of the light microscope. Chromosome preparations in leukaemia can be of poor quality

so that accurate analysis is difficult. Cytogenetic analysis is insensitive for monitoring residual disease since conventionally only 20 metaphases are examined.

FISH and comparative genomic hybridization

Cytogenetic analysis can be supplemented by *in situ* hybridization techniques, particularly fluorescence *in situ* hybridization (FISH), to identify specific DNA sequences that are amplified, deleted or involved in chromosomal rearrangements. FISH can be applied to cells in metaphase or interphase. FISH also has the advantage of speed; results can be obtained within a single working day. Since specific DNA sequences are identified, FISH is a molecular genetic technique but, since chromosomes can be counterstained and thus their size and shape permits them to be recognized, clearly it also makes use of traditional cytogenetic techniques. The development of FISH has led to a further specialized terminology and abbreviations [103] (Table 2.14). The copy number of specific loci is shown by the name of the locus coupled with a multiplication sign and the number of copies. The juxtaposition of loci is indicated by the abbreviation *con*. For example, interphase FISH analysis of cells from a patient with t(8;21)(q22;q22) could be recorded as .nuc ish (ETOx2),(RUNX1x2)(ETO con RUNX1 x1), ETO designating a probe for *RUNX1T1*. This .nuc ish follows the conventional karyotype. Labelled probes used can be: (i) centromeric probes, which specifically identify each chromosome by means of chromosome-specific α satellite sequences at its centromere; (ii) probes for specific DNA sequences usually corresponding to genes, either oncogenes or tumour suppressor gene sequences; or (iii) 'whole chromosome paints', which are libraries of chromosome-specific probes that hybridize to sequences extending over the whole chromosome. Translocations can be recognized by a single-colour probe that spans the expected breakpoint region and is therefore split in a given translocation. The cells therefore have three signals rather than the usual two. Alternatively, a dual-colour probe can be used. This technique incorporates two differently labelled probes that flank the gene. In normal cells the red plus green signals give a yellow (flanked by red and green) fusion signal; when a translocation splits the gene the red and green signals are separated and appear on different chromosomes (Fig. 2.11). Split signal FISH is very useful when a single oncogene has multiple partners, e.g. the *MLL* gene in acute

Table 2.14 Some symbols, terminology and abbreviations used in describing fluorescence *in situ* hybridization (FISH).

–	Minus sign, absent signal from a specific chromosome
+	Plus sign, signal present on a specific chromosome
++	Two plus signs, signal duplicated on a specific chromosome
x	Multiplication sign, precedes number of signals, e.g. –x2 indicates signals missing on the two homologous chromosomes
.	Full stop, separated cytogenetic results from *in situ* hybridization results
;	Semicolon, separates probes on different derivative chromosomes, e.g. 46,XX,t(9;22)(q34;q11.2)[20].ish t(9;22)(ABL1–;BCR+,ABL1+ means that no ABL1 signal is detected on the der(9) whereas on the der(22) there is both an ABL1 signal and a BCR signal; note also the use of the full stop between the cytogenetic results and the ish results
con	With, signals are juxtaposed
dim	Diminished signal intensity
enh	Enhanced signal intensity
FISH	Fluorescence *in situ* hybridization
ish	*In situ* hybridization
nuc ish	Nuclear or interphase *in situ* hybridization, e.g.: nuc ish(ABL1,BCR) x2[100] indicates that 100 interphase nuclei have been assessed and all have the normal number of BCR and ABL1 signals nuc ish(ABL1 x2),(BCR x2),(ABL1 con BCR x1)[100] indicates that the normal number of signals are present but one BCR and one ABL1 signal are juxtaposed as a result of a t(9;22)
sep	Separated, signals that are expected to be juxtaposed have separated
wcp	Whole chromosome paint

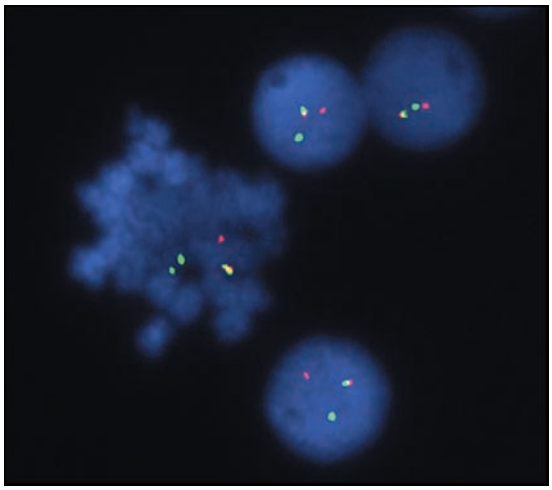


Fig. 2.11 Fluorescence *in situ* hybridization (FISH) using a dual-colour, break-apart probe for *RARA*. As a result of t(15;17)(q22;q12) in a patient with acute promyelocytic leukaemia the *RARA* gene has been split. Normal cells would have two red-yellow-green fusion signals whereas these leukaemic cells have one normal fusion signal and separate red and green signals, on derivative chromosomes 17 and 15 respectively. (By courtesy of Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

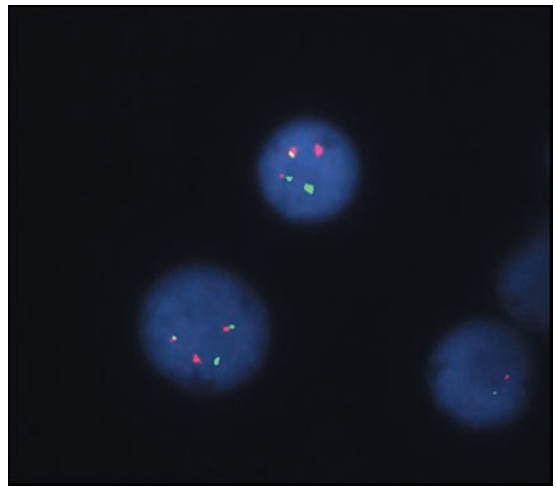


Fig. 2.12 FISH using a dual-colour, dual-fusion technique. Normal cells would have two red signals (*PML* on chromosome 15) and two green signals (*RARA* on chromosome 17). As a result of t(15;17)(q22;q12) in a patient with acute promyelocytic leukaemia one allele of each gene has been split with subsequent rearrangement leading to formation of *PML-RARA* and *RARA-PML* fusion genes. As a result the leukaemic cells have a red signal, a green signal and two fusion signals. One of the fusion signals is red-yellow and the other has red and green signals in close proximity. (By courtesy of Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

leukaemia. In an alternative technique, dual-colour dual-fusion FISH, the two probes (one red and one green) span the breakpoints on the two chromosomes likely to be involved; when a translocation occurs both probes are split and two new fusion signals are produced on each of the involved chromosomes (Fig. 2.12). Probes used for FISH can be designated by the clone name, or the locus (from the Genome Database) or the gene name (using approved terminology). Although use of the clone names is preferred because it is more scientifically accurate, use of the locus or gene name is more intelligible to clinical staff; it is thus sensible to use the clone name in giving the results but include the locus or gene name in the interpretative comments.

Kits are available that permit multiple FISH hybridizations (e.g. 5–10) spacially separated on a single slide. The panel can be selected to cover B-lineage ALL, T-lineage ALL, MDS/AML or CLL.

Modified FISH techniques, such as multiplex FISH (M-FISH) and spectral karyotyping (SKY) that permit the identification of each chromosome pair in a different colour are very useful in elucidating the nature of complex rearrangements (Fig. 2.13).

Comparative genomic hybridization (CGH) is another modification of standard cytogenetic techniques, which can be used to demonstrate areas of chromosomal gain or loss in neoplastic cells. The principle of this technique is that normal and test DNA are labelled with two different fluorochromes and the mixture is then hybridized to normal human metaphase chromosomes, so that under- or over-representation of sequences of DNA can be detected as areas where one or other colour dominates; an abnormality must be present in a significant proportion of tumour cells (e.g. >50%) to be detected and balanced aberrations such as translocations and inversions will not be detected. CGH is more applicable to research than to routine diagnosis. More recently, high resolution array-based CGH and single nucleotide polymorphism (SNP) arrays have superseded chromosome-based

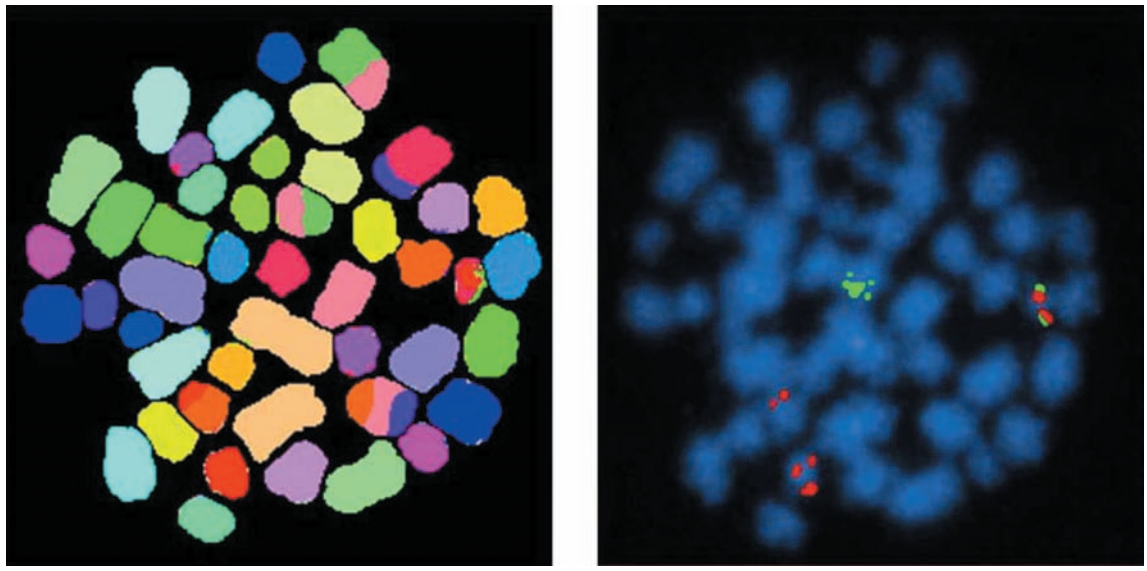


Fig. 2.13 FISH of chromosomes of a patient with ALL and the common translocation, t(12;21)(p13;q22), associated with a complex karyotype: (a) metaphase by multicolour FISH; (b) the same metaphase hybridized with probes to *ETV6-RUNX1* (green-red) showing the gene fusion (red and green together), normal chromosome 12 (green) and extra copies of *RUNX1*. In this patient there are complex translocations shown by the shift in colour between the chromosomes indicating a complex series of chromosomal rearrangements between chromosomes 5, 9, 12, 18 and 21. (By courtesy of the Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne.)

CGH. These new approaches use DNA clones or oligonucleotides and have demonstrated significant focal copy number changes in leukaemia.

Problems and pitfalls in FISH analysis

The main disadvantage of FISH analysis is that, in contrast to cytogenetic analysis, usually only the abnormalities that are specifically sought are detected, e.g. analysis for *BCR-ABL1* fusion in imatinib-treated chronic granulocytic leukaemia may indicate a declining neoplastic population but will fail to detect a new clonal abnormality in Ph-negative cells. Nevertheless FISH is very useful in searching for a restricted range of abnormalities in cell populations, such as those of CLL, that do not readily enter mitosis. FISH analysis is insensitive for the detection of a small clone in post-treatment follow-up.

Molecular genetic analysis

Molecular genetic analysis may be based on analysis of DNA by the polymerase chain reaction (PCR) or on analysis of RNA by reverse transcriptase PCR (RT-PCR). DNA analysis by the Southern blot technique has now been superseded, for diagnostic purposes, by PCR. These and other molecular techniques are summarized in Table 2.15. Sensitivity of PCR is increased by the technique of nested PCR. Analysis can be made quantitative by the use of real-time quantitative PCR (RQ-PCR). Gene expression profiling by microarray analysis is likely to become increasingly important in the analysis of leukaemic cells, both for research and as an aid in determining diagnosis and prognosis in relation to specific gene signatures. It permits the expression of thousands of genes to be studied simultaneously but it is also possible to design smaller panels to ask specific questions.

Problems and pitfalls in molecular genetic analysis

Because of its great sensitivity, contamination is a potential problem in molecular analysis and techniques must be meticulous. It is also necessary to be aware that a low level of a number of fusion genes characteristic of specific neoplasms can be detected in healthy individuals or in non-neoplastic tissues. As for FISH analysis, molecular analysis will detect

Table 2.15 A summary of molecular genetic techniques used in the investigation of leukaemia.

Technique	Principle	Application
Molecular cytogenetic techniques		
Fluorescence *in situ* hybridization (FISH)	Chromosomes or specific DNA sequences are identified by a probe bound to a fluorochrome; applicable to chromosomes in metaphase and, to a lesser extent in interphase	Detection of numerical abnormalities (monosomies, trisomies, hyperdiploidy); identification of translocations, detection of amplification of oncogenes or loss of either tumour suppressor genes or the normal allele corresponding to a gene contributing to a fusion gene (e.g. loss of *ETV6* in t(12;21)-associated acute lymphoblastic leukaemia)
Multiplex FISH (M-FISH)	Multicolour FISH using five fluorochromes in different combinations so that all chromosomes pairs can be identified simultaneously; five separate fluorochrome images are captured	Clarification of complex karyotypes
Spectral karyotyping (SKY)	Multicolour FISH using five fluorochromes and capturing a single image; combinations of fluorochromes are recognized by their spectral signature	Clarification of complex karyotypes
Comparative genomic hybridization (CGH)	Labelled patient and normal DNA, differentially labelled with fluorochromes, are hybridized to normal metaphases	Identification of the region of a chromosome where there is a gain or a loss; can be used for indicating the likelihood of amplified oncogenes, e.g. in double minute chromosomes
Molecular genetic techniques		
Southern blot	DNA is digested by restriction endonucleases; the restriction fragments created are separated by gel electrophoresis following which they are blotted onto a membrane; a radioactive probe is then used to identify the DNA sequence of interest on a fragment of a specific size	Detection of rearrangement of a gene, e.g. an immunoglobulin or T-cell receptor gene (for demonstration of clonality) or rearrangement of an oncogene such as *MLL* which has multiple partners
Polymerase chain reaction (PCR)	A method of *in vitro* amplification of a defined DNA target that is flanked by regions of known sequence; to distinguish it from RT-PCR, this technique may be referred to as genomic PCR or DNA PCR	Detection of rearrangement of a gene, e.g. an immunoglobulin or T-cell receptor gene (for demonstration of clonality) or an oncogene – a much more sensitive technique than Southern blot analysis
Reverse transcriptase PCR (RT-PCR)	An *in vitro* method for reverse transcription of RNA followed by amplification of complementary DNA	Analysis of genes that are too long for analysis by a standard genomic PCR
Multiplex PCR	Simultaneous application of a number of pairs of primers so that any of a number of possible mutations can be identified	Simultaneous screening for a number of leukaemia-related mutations
Real-time quantitative PCR (RQ-PCR)	A quantitative PCR technique in which there is displacement of a fluorogenic product-specific probe which is degraded during the reaction, generating a fluorescent signal	Quantification of the amount of a specific DNA sequence present – useful for monitoring minimal residual disease

Table 2.15 (*Continued*)

Technique	Principle	Application
Gene expression profiling	A technique for studying the expression of very large numbers of genes by means of quantification of the binding of RNA from the tissue in question to arrays of dots (DNA or oligonucleotides) in multiple rows on a glass slide, each dot representing a single gene	Identification of the genomic positions where there is a gain or a loss; potential uses include: (i) as a marker of cytogenetic/molecular genetic subtypes; (ii) detection of expression of specific genes, e.g. *FLT3*; (iii) prediction of response in general or response to specific treatments; and (iv) prediction of prognosis
Molecular histological techniques		
In situ hybridization for detection of messenger RNA (mRNA)	A labelled probe detects specific mRNA, e.g. mRNA for κ or λ or mRNA of an oncogene such as cyclin D1 (*CCND1*)	Establishment of clonality or confirmation that an oncogene is expressed
Immunohistochemistry for detection of a gene product	An antibody (polyclonal or monoclonal) is raised to the protein product of a specific gene, e.g. an oncogene or a tumour suppressor gene	Demonstration that a normal or mutant oncogene or tumour suppressor gene has a protein product in a specific cell (e.g. ALK or p53) or that a protein product has an abnormal distribution (e.g. PML protein in acute promyelocytic leukaemia and ALK in anaplastic large cell lymphoma)

only the specific abnormalities that are sought so that investigations must be targeted appropriately. Molecular analysis that depends on RNA rather than DNA requires a high quality sample since degradation occurs on storage.

The role of cytogenetic and molecular genetic analysis in haematological neoplasms

Examples indicating the type of information that can be derived from classical cytogenetic analysis in known or suspected haematological neoplasms are given in Table 2.16. When the karyotype of bone marrow cells is studied, some cells show random abnormalities, which need to be distinguished from a non-random or consistent abnormality that indicates the presence of an abnormal clone. For this reason, according to the International System of Nomenclature [106], a clone is considered to be present if two cells show the same structural change or additional chromosomes or if three cells show the same missing chromosome. However, it should be noted that loss of the Y chromosome in men is usually an age-related change, rather than being evidence of a neoplastic clone. Trisomy 15 of bone marrow cells, either isolated or associated with –Y, has also been found *not* to be predictive of evolution to an overt haematological neoplasm [107].

Further information can be derived from molecular genetic analysis (Table 2.17). The purpose of molecular genetic analysis may be either the establishment of clonality, by detection of rearrangement of either an *IGH* or *TCR* (T-cell receptor) locus, or the identification of a molecular rearrangement characteristic of a specific neoplasm. The results of cytogenetic and molecular genetic analysis should be interpreted only in the light of the cytological features since the same chromosomal abnormalities may be found in both acute and chronic leukaemia, in both AML and ALL, or in both AML and MDS. However, there are some cytogenetic abnormalities that identify a subtype of AML or non-Hodgkin lymphoma with such high specificity that the subtype may be defined more accurately by the karyotype than by the morphology.

Problems and pitfalls in cytogenetic and molecular genetic analysis

Cytogenetic analysis should be done on bone marrow cells, when possible, since use of peripheral blood cells can lead to an abnormality being missed.

Table 2.16 Some examples demonstrating the role of cytogenetic analysis in haematological neoplasms.

- Recognition of an underlying constitutional abnormality that predisposes to AML such as Down syndrome (trisomy 21 or equivalent), Fanconi anaemia (susceptibility to clastogenic agents), familial monosomy 7 syndrome, familial t(7;20)(p?p?) [104], familial t(3;6)(p14;p11) [105]
- Recognition of subtypes of AML with differing prognosis requiring differing therapeutic approaches, including confirmation of acute promyelocytic leukaemia by demonstration of t(15;17)(q22;q12)
- Confirmation of AML rather than MDS in patients with a low blast percentage, e.g. in patients with t(8;21)(q22;q22) and inv(16)(p13q22)
- Identification in AML of specific chromosomal rearrangements known to be associated with specific fusion genes, in order to indicate which molecular techniques are likely to be useful for assessing minimal residual disease
- Recognition of therapy-related AML, following either alkylating agents or topoisomerase II-interactive drugs
- Recognition of good or bad prognostic groups in B-lineage ALL, e.g. high hyperdiploidy, t(9;22), t(4;11)
- Confirmation of a diagnosis of chronic myelogenous leukaemia by demonstration of t(9;22)(q34;q11.2)
- Confirmation of a diagnosis of chronic eosinophilic leukaemia by identification of a clonal abnormality
- Identification of chronic eosinophilic leukaemia or other myeloid neoplasm associated with rearrangement of *PDGFRB* by demonstration of a clonal rearrangement with a 5q31~33 breakpoint
- Identification of chronic eosinophilic leukaemia or other lymphoid or myeloid neoplasm associated with rearrangement of *FGFR1* by demonstration of a clonal rearrangement with an 8p11 breakpoint
- Confirmation of a diagnosis of mantle cell lymphoma by demonstration of t(11;14)(q13;q32) or a variant translocation
- Confirmation of a diagnosis of follicular lymphoma by demonstration of t(14;18)(q32;q21) or a variant translocation
- Confirmation of a diagnosis of Burkitt lymphoma by demonstration of t(8;14)(q24;q32) or a variant translocation
- Furthering knowledge of leukaemogenesis, e.g. by identifying sites of possible oncogenes, demonstration of the leukaemic nature of transient abnormal myelopoiesis of Down syndrome

AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome.

Table 2.17 Some examples demonstrating the role of molecular genetic analysis in haematological neoplasms.

- Recognition of subtypes of AML with differing prognosis requiring differing therapeutic approaches, including confirmation of AML of M3 or M3 variant subtype by demonstration of *PML-RARA* fusion gene, detection of the good prognosis *RUNX1-RUNX1T1* (*AML1-ETO*) and *CBFB-MYH11* fusion genes and detection of the poor prognosis *BCR-ABL1* fusion gene
- Recognition of prognostically significant molecular abnormalities in AML that cannot be detected by conventional cytogenetic analysis, e.g. detection of internal tandem duplication of *FLT3* (poor prognosis) or of *NPM1* or *CEBPA* mutation (good prognosis)
- Confirmation of AML rather than MDS in patients with a low blast percentage, e.g. in patients with *RUNX1-RUNX1T1* and *CBFB-MYH11* fusion genes
- Recognition of therapy-related AML, e.g. by demonstration of *MLL* rearrangement following exposure to topoisomerase II-interactive drugs
- Recognition of subtypes of ALL associated with cryptic chromosomal rearrangements, e.g. by detection of *ETV6-RUNX1* associated with cryptic t(12;21)(p13;q22)
- Recognition of prognostically significant cytogenetic subgroups in patients with B-lineage ALL and failed or normal cytogenetic analysis
- Confirmation of a diagnosis of Ph-positive ALL or chronic myelogenous leukaemia by demonstration of *BCR-ABL1*
- Confirmation of a diagnosis of a myeloid or lymphoid neoplasm associated with rearrangement of *PDGFRA* by demonstration of *FLIP1L1-PDGFRA*
- Monitoring of minimal residual disease, either by detection of the product of a fusion gene or by detection of overexpression of a gene, e.g. *WT1*, which is often overexpressed in AML
- Furthering knowledge of leukaemogenesis and of normal haemopoiesis, e.g. by identification of oncogenes and by demonstration of their role in normal haemopoiesis; identification of the mechanism of leukaemogenesis; demonstration of the intrauterine origin of some cases of AML in infants and children

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; MDS, myelodysplastic syndrome.

Even cytogenetic analysis of bone marrow cells may yield no analysable metaphases or only normal metaphases despite a clone of cytogenetically abnormal cells being present. This is well recognized in acute leukaemia and means that cytogenetic analysis has to be supplemented by molecular analysis when it is particularly important to recognize or exclude a specific abnormality. Such analysis may be confined to patients with absent or only normal metaphases. In chronic B-lineage leukaemias, leukaemic cells may not readily enter mitosis so that there are no metaphases of leukaemic cell origin for analysis. It is possible to use mitogens with some specificity for B cells but in chronic lymphocytic leukaemia the solution is usually to use FISH rather than classical cytogenetic analysis.

Interpretative errors include misinterpretation of a constitutional abnormality or −Y as evidence of clonality. When necessary, cytogenetic analysis can be carried out on stimulated peripheral blood lymphocytes to determine the constitutional karyotype. Misinterpretation of −Y by clinical staff can be avoided by appropriate interpretative comments from the cytogeneticist.

Molecular genetic analysis is often used to provide evidence of clonality by detecting the rearrangement of *IGH* or TCR loci. However, not all B and T neoplasms arise from a cell that has rearranged these loci. It should also be noted that, in dealing with acute leukaemias, rearrangement of a locus does not necessarily indicate that the neoplastic cells have arisen from that lineage.

In molecular genetic analysis, evidence of a fusion gene may not be found if primers appropriate for a variant breakpoint are not used or if nested PCR is not used when it is indicated. For example, in chronic eosinophilic leukaemia, detection of *FIP1L1-PDGFRA* often requires nested RT-PCR.

Cytogenetic and molecular genetic analysis in acute leukaemia

The role of cytogenetic and molecular genetic analysis in acute leukaemia includes: (i) demonstration of a specific abnormality that may be relevant to classification, prognosis and choice of treatment; (ii) demonstration of an abnormality that indicates that a leukaemia is likely to be therapy related; (iii) provision of prognostic information following response to initial chemotherapy; (iv) identification of an abnormality that can be used for monitoring of MRD; and (v) identification of leukaemia in *donor* cells following haemopoietic stem cell transplantation [108]. The cytogenetic and molecular genetic techniques now available include: (i) standard cytogenetic analysis; (ii) FISH; (iii) PCR; (iv) RT-PCR; (v) RQ-PCR; and, potentially, (vi) gene expression profiling. It could be speculated that in the future the diagnosis of AML and of B-lineage and T-lineage ALL might be made by microarray analysis [109,110].

Minimal residual disease

Minimal residual disease can be monitored by molecular genetic techniques including PCR, RT-PCR and RQ-PCR. Cytogenetic analysis is of much less use since it is possible to examine only a relatively small number of metaphases (conventionally only 20). For technical reasons, FISH is also relatively insensitive in the monitoring of MRD, despite the considerably larger number of cells that can be examined. When a fusion transcript is present it can be used for monitoring, but in patients lacking a detectable fusion gene an alternative technique is needed. The *WT1* gene, which is expressed at only a low level in normal bone marrow, is overexpressed in about 85% of patients with AML. Detection of such overexpression, which is about 3 logs higher than in normal cells, by RQ-PCR is potentially of use in these patients. In the case of ALL, a rearranged *IGH* or *TCR* locus that is specific for the leukaemic clone can often be used. It is not possible to find a molecular marker in all cases of acute leukaemia but it is generally possible to find either an immunophenotypic marker or a molecular marker.

Cytogenetic and molecular genetic analysis in acute myeloid leukaemia

Cytogenetic analysis In AML, the abnormal clone of cells may include the granulocyte/monocyte, erythroid and megakaryocyte lineages or be restricted to the granulocytic/monocytic lineage.

Successful cytogenetic analysis is more often possible on a bone marrow aspirate than on peripheral blood cells. Bone marrow aspiration is therefore recommended for this purpose, even if it is obvious from the peripheral blood that the patient has acute leukaemia. Cytogenetic analysis at diagnosis is used

to assess prognosis and therefore influences treatment decisions. In addition, in one study cytogenetic analysis at day 21 from the start of treatment was found to give independent prognostic information in patients with an initially abnormal karyotype, cytogenetic response correlating with longer disease-free survival; known good prognosis karyotypes were excluded from the study [111].

Occasionally karyotypic evidence suggests the presence of two independent clones. Although this may occur, particularly when the bone marrow has been exposed to mutagenic influences, evidence from the analysis of glucose-6-phosphate dehydrogenase (G6PD) alloenzymes and from DNA analysis suggests that in some patients apparently independent clones are subclones derived from a single parent clone that was cytogenetically normal.

Cytogenetic analysis provides some of the evidence that AML can be divided into two broad groups. Cases associated with balanced translocations show a three- to four-fold increase in incidence with increasing age whereas cases associated with complex abnormal karyotypes (defined in the study as having at least three numerical or structural abnormalities) show an almost 30-fold increase [112]. The former group comprises mainly cases of *de novo* leukaemia whereas the latter group includes myelodysplasia-related AML.

Molecular genetic analysis Increasingly, patients with AML are investigated by molecular genetic as well as cytogenetic techniques [113]. Molecular techniques are applicable to diagnosis and classification, give prognostic information and can be used for the monitoring of MRD. The recurrent cytogenetic abnormalities associated with specific subtypes of acute leukaemia can be detected by FISH, by genomic PCR (DNA amplified) and by RT-PCR (messenger RNA reverse transcribed and the complementary DNA amplified). Multiplex PCR is a useful way to screen cases for the most important translocations by using multiple pairs of primers for the identification of specific rearrangements. For example, cases of AML can be simultaneously screened for inv/t(16), t(15;17) and t(8;21). Techniques can be adapted to screen for very large numbers of fusion products [114].

Molecular genetic analysis can be used not only to identify specific subtypes of AML but also to detect mutations such as those in *RAS* genes (*NRAS* or *KRAS*) or *FLT3* that are not closely related to subtype but may be of prognostic significance and may indicate the likelihood of therapeutic response to specific inhibitors of the gene product. *FLT3*-internal tandem duplication (*FLT3*-ITD) is found in more than a quarter of patients with AML and is indicative of a considerably worse prognosis [115]. Overall, this poor prognosis is not improved by transplantation [116]. *FLT3* mutations are less common in children than in adults, being found in only 10–15% of paediatric patients [117]. Further molecular indicators of worse prognosis include partial tandem duplication of *MLL* (*MLL*-PTD), *WT1* mutation, overexpression of *EVI1* (which may indicate cryptic chromosomal rearrangement) and overexpression of *BAALC*, *MN1*, *ERG* and *WT1*.

Molecular genetic analysis has been used to demonstrate the intrauterine origin of some cases of AML associated with t(8;21) [118].

The technique of microarray analysis is becoming increasingly important in AML. The gene expression pattern differs between AML subtypes, e.g. AML associated with *RUNX1-RUNX1T1* (*AML1-ETO*), *PML-RARA*, *CBFB-MYH11*, *MLL* rearrangement, *CEBPA* mutation, *EVI1* overexpression and *FLT3*-ITD, but there are also clusters of cases that share a gene expression pattern that does not correlate with known cytogenetic/molecular genetic subtypes [119–122]. Microarray analysis can be used to identify expression of prognosis-associated genes in paediatric [123] and adult [121,124] AML. There is a potential to use microarray analysis not only for diagnosis, classification and prognostication but also for stratification and selection of targeted treatment.

Integration of cytogenetic and molecular genetic data Cytogenetic and molecular genetic analyses are complementary in identifying the defining abnormality of a particular subtype of AML. However, there are likely to be other independent abnormalities that also contribute to the disease phenotype. It has been postulated that the development of AML requires the coexistence of two types of mutation, a class I mutation that leads to a proliferation/survival advantage and a class II mutation that interferes with differentiation [125]. However, there may be some fusion genes that are in themselves sufficient

Table 2.18 Type I and type II mutations that can interact in the pathogenesis of acute myeloid leukaemia [127–129].

Class II mutation (interferes with differentiation)	Class I mutation (conveys proliferation or survival advantage)*	Effect of class I mutation on prognosis
RUNX1-RUNX1T1 resulting from t(8;21)(q22;q22)	*KIT* mutation (12–47% of cases) *NRAS* (*c.* 10%) *FLT3*-ITD (*c.* 4%)	Worse [127,128] No difference [129]
CBFB-MYH11 resulting from inv(16)(p13q22) or t(16;16)(p13;q22)	*NRAS* (*c.* 30–40%) *FLT3*-ITD (*c.* 7%) *KIT* mutation (22–47% of cases)	No difference [129] No difference [127] or worse [128]
PML-RARA resulting from t(15;17)(q22;q12)	*FLT3*-ITD (*c.* 30%) *NRAS* (*c.* 2%)	Possibly worse
CEBPA mutated	*FLT3*-ITD	Worse
NPM1 mutated	*FLT3*-ITD	Worse

* Reported incidence in subtype shown in brackets.

to cause the leukaemic phenotype, such *FUS-ERG*, *MLL-MLLT1*, *MLLT3-MLL* and *MYST3-NCOA2* [126]. There is a non-random association between specific class I and class II mutations. Since the presence or absence of a specific class I mutation can influence prognosis and can potentially influence choice of treatment the identification of both types of mutation may become increasingly important. Some of these non-random associations are shown in Table 2.18. Their significance will become clearer when individual subtypes of AML are dealt with in Chapter 3.

Cytogenetic and molecular genetic abnormalities in relation to morphological subtypes of acute myeloid leukaemia and prognosis With current techniques, 70–80% of patients with AML are found to have non-random (clonal) cytogenetic abnormalities [103,130], many of which are recurrent (i.e. they have been reported in a more than one patient, often occurring in a significant proportion of patients) [131]. Overall the commonest cytogenetic abnormality is trisomy 8 with anomalies of chromosome 7 being in second place. Some chromosomal anomalies, such as trisomy 8 and trisomy 21, are found in all morphological subtypes and in both secondary and *de novo* leukaemia; they are not related to any readily apparent morphological or clinical features. Other anomalies, including t(15;17), t(8;21) and t or inv(16), have a strong association with a particular morphological subtype; they rarely occur in secondary leukaemia, and erythroid and megakaryocyte dysplasia are not usually a feature. It is possible that, in this group of anomalies, the leukaemia has arisen in a lineage-restricted stem cell. Other anomalies such as t(6;9)(p23;q34), t(1;7)(q10;p10), t(3;3)(q21;q26.2) and inv(3)(q21q26.2) occur in multiple morphological subtypes and in MDS as well as in both *de novo* and secondary (irradiation or cytotoxic-drug-related) leukaemias. It is likely that the association of such translocations with bi- or trilineage myelodysplasia and with multiple morphological categories indicates that the leukaemia has arisen in a multipotent stem cell, which has preserved its capacity to differentiate into cells of various lineages. Other anomalies involving predominantly loss of chromosomal material (such as −5, 5q−, −7, 7q−) show a similar lack of relationship to morphological subtypes but an association with myelodysplastic features and with therapy-related MDS and secondary AML. Many patients with AML have more than one karyotypic abnormality. Complex abnormalities are particularly characteristic of erythroleukaemia, therapy-related AML and AML arising in patients with previous MDS, a myeloproliferative neoplasm or a myelodysplastic/myeloproliferative neoplasm. Chromosomal abnormalities that are strongly associated with characteristic clinical and morphological features are often termed specific, whereas those that are not are termed non-specific.

Chromosomal abnormalities have been found to have independent prognostic significance in AML

Table 2.19 Prognostic significance of haematological, cytogenetic and molecular genetic abnormalities in acute myeloid leukaemia [130,132–138].

Nature of criterion	Good	Intermediate	Adverse
Clinical and haematological	WBC no greater than 20×10^9/l Early complete remission		WBC greater than 100×10^9/l
Cytogenetic	t(8;21)(q22;q22) t(15;17)(q22;q12) inv(16)(p13.1q22) t(16;16)(p13.1;q22)	Neither good prognosis nor poor prognosis abnormalities present	–5, –7, del(5q), add(5q), abnormal 3q, inv(3)(q21q26.2), t(3;3)(q21;q26.2), t(10;11)(p13;q23), abnormal 17p plus other abnormality, –17, t(6;11)(q27;q23), t(9;22)(q34;q11.2), add(7q), t(6;9)(p23;q34), t(1;7)(q10;p10) Complex karyotype (defined as three or more or five or more unrelated abnormalities) Monosomy of two autosomes or of one autosome plus a structural abnormality
Molecular	*NPM1* mutated (in the absence of *FLT3*-ITD) *CEBPA* mutated		*FLT3*-ITD Mutation of *WT1** *MLL*-PTD* Overexpression of *BAALC**, *ERG**, *WT1*, *EVI1*, *MN1** *KIT* mutation (in cases with t(8;21) or t/inv(16))

WBC, white blood cell count.
* In patients with normal cytogenetic analysis.

although the prognostic ranking has not been identical in different series of patients [130,132–138] (Table 2.19). Several very large clinical trials, under the auspices of the UK Medical Research Council, have established and validated prognostic grouping on the basis of cytogenetic abnormalities. In children and adults under the age of 55 years, a favourable prognosis was associated with inv(16), t(8;21) and t(15;17), whereas an adverse prognosis was associated with –5, –7, del(5q) (also known as 5q–), 3q abnormalities and a complex karyotype (five or more unrelated abnormalities); del(7q) (also known as 7q–) was not prognostically adverse unless it was part of a complex karyotype [136]. Patients not falling into either the favourable or the adverse group were assigned to an intermediate category. In older patients (median age 66 years), observations were similar except that the complex karyotype group had a significantly worse outcome than patients with –5, –7, del(5q) or 3q abnormalities and the latter group was therefore re-assigned to the intermediate prognosis group. This prognostic categorization now determines treatment selection in UK trials. A group of patients with autosomal monosomies (see Table 2.19) have been found to have a highly adverse prognosis and if they are removed from the complex karyotype group its significance is not so adverse [137].

Cytogenetic and molecular genetic analysis in acute lymphoblastic leukaemia

Cytogenetic analysis The role of cytogenetic analysis in ALL is similar to its role in AML [139]. In addition, it can be important in distinguishing Burkitt lymphoma from cases of precursor-B-cell ALL with FAB L3 cytological features. The results of cytogenetic analysis are of prognostic significance. Best prognosis is associated with high hyperdiploidy (defined as 51–65 chromosomes or detected by demonstrating simultaneous trisomy for chromosomes 4, 10 and 17) and with t(12;21)(p13;q22) (*ETV6-RUNX1* being detected by molecular means). Worst prognosis is associated with t(9;22), *MLL* rearrangement and hypodiploidy (fewer than 44 chromosomes).

Occasionally cytogenetic analysis has yielded new information on leukaemogenesis, indicating the intrauterine origin of some cases of B-lineage childhood ALL associated with hyperdiploidy t(12;21) or t(4;11). Intrauterine origin appears to be much less frequent in the case of T-lineage ALL but has been described in one child with a *TCRD-LMO2* fusion [140].

Molecular genetic analysis Molecular genetic analysis has been important in the recognition of subtypes of ALL associated with a recurring molecular genetic abnormality without any cytogenetic correlate. It is essential for the identification of an *ETV6-RUNX1* fusion gene indicative of a cryptic t(12;21)(p13;q22).

In infants and children, molecular genetic analysis has been of value in determining whether leukaemia has its origin in intra- or extrauterine life. Using such techniques to demonstrate specific fusion genes, comparison of cord blood samples with samples taken after the development of leukaemia have shown an intrauterine origin for some cases of ALL associated with t(4;11), t(12;21) and t(1;19) as well as for other T- and B-lineage cases without any of these abnormalities [118]. Cases of ALL can be simultaneously screened by multiplex PCR for t(9;22), t(4;11) and t(1;19).

High resolution array-based techniques are yielding new information in ALL. They have shown mutations and deletions involving the B-cell differentiation genes to be highly significant in B-lineage ALL. Specifically, one of these genes, *IKZF1*, has been linked to a poor prognosis. With gene expression profiling it is possible to recognize characteristic gene expression signatures associated with t(1;19) (*TCF3-PBX1*, previously known as *E2A-PBX1*), t(12;21) (*ETV6-RUNX1*), t(9;22) (*BCR-ABL1*), t(8;14) (*MYC* translocated), *MLL* rearrangement and hyperdiploidy [110,120,141]. Interestingly, cases with t(12;21) and with high hyperdiploidy have some gene expression characteristics in common, suggesting a possible common biological pathway responsible for the leukaemic phenotype [142]. Microarray analysis can also permit recognition of clusters of cases that differ from, and overlap with, known cytogenetic/molecular genetic categories but which may nevertheless have biological significance. Thus two clusters can be recognized within T-lineage ALL and seven clusters within B-lineage ALL, which do not relate closely to cytogenetic subtypes [143]. A new *BCR-ABL1*-like group has been recognized from its characteristic gene expression signature. This novel group of childhood ALL has been associated with a poor prognosis. Drug-resistant ALL can be distinguished from fully sensitive ALL with a correlation with treatment outcome being observed [144]. It may even be possible to predict which patients within a specific subtype of ALL (*ETV6-RUNX1* positive) are likely to develop therapy-related AML [122].

Conclusions

The classification of leukaemia and related disorders is increasingly integrating cytogenetic and molecular genetic analysis with morphology and immunophenotyping. This is leading to a more complex but more clinically meaningful classification of these disorders, which in turn is leading to improved patient outcome as treatment becomes more targeted.

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THREE

ACUTE LEUKAEMIA: INTEGRATION OF MORPHOLOGICAL, IMMUNOPHENOTYPIC AND GENETIC INFORMATION AND THE WHO CLASSIFICATION

Leukaemia Diagnosis, 4th edition. By Barbara J. Bain. Published 2010 by Blackwell Publishing.

Introduction

Cytology, possibly supplemented by cytochemistry, is the starting point for the diagnosis and classification of the acute leukaemias but crucial information is also yielded by immunophenotyping and by cytogenetic and molecular genetic analysis. An ideal classification must incorporate all these elements. The 2008 World Health Organization (WHO) classification follows this approach, which had previously been suggested by others in the MIC (Morphology, Immunophenotype, Cytogenetics) and MIC-M (Morphology, Immunophenotype, Cytogenetics, Molecular genetics) classifications [1–3].

The application of cytology and cytochemistry to haematological neoplasms was discussed in Chapter 1 and cytogenetic and genetic analysis in Chapter 2. This chapter brings together all these techniques in an integrated approach, based on the WHO classification.

The WHO classification of acute leukaemias

The 2008 WHO classification of acute leukaemias is part of a broader classification of tumours of haemopoietic and lymphoid tissues [4]. It builds on the work of the French–American–British (FAB) group and on earlier WHO classifications published in 1999 and 2001. The principle that has been followed is that real entities should be recognized based, as far as possible, on the phenotype (morphological and immunological) and on the underlying genetic abnormalities that determine disease

Table 3.1 The WHO classification of acute myeloid leukaemia (AML) [5].

Therapy-related myeloid neoplasms
AML with recurrent genetic abnormalities*
AML with t(8;21)(q22;q22); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13;q22); *CBFB-MYH11*
AML with t(15;17)(q22;q12); *PML-RARA*
AML with t(9;11)(p22;q23); *MLLT3-MLL*
AML with t(6;9)(p23;q34); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *RPN1-EVI1*
AML with t(1;22)(p13;q13); *RBM15-MKL1*
Provisional entity: AML with mutated *NPM1*
Provisional entity: AML with mutated *CEBPA*
AML with myelodysplasia-related changes
AML not otherwise categorized
AML with minimal differentiation
AML without maturation
AML with maturation
Acute myelomonocytic leukaemia
Acute monoblastic/monocytic leukaemia
Acute erythroid leukaemia
Pure erythroid leukaemia
Erythroleukaemia, erythroid/myeloid
Acute megakaryoblastic leukaemia
Acute basophilic leukaemia
Acute panmyelosis with myelofibrosis
Myeloid sarcoma
Myeloid proliferation related to Down syndrome
Transient abnormal myelopoiesis
Myeloid leukaemia associated with Down syndrome
Blastic plasmacytoid dendritic cell neoplasm

* If therapy-related cases are found to have these recurrent cytogenetic abnormalities this should be noted but the cases are categorized as therapy-related AML or myelodysplastic syndrome, not as AML with recurrent cytogenetic abnormalities.

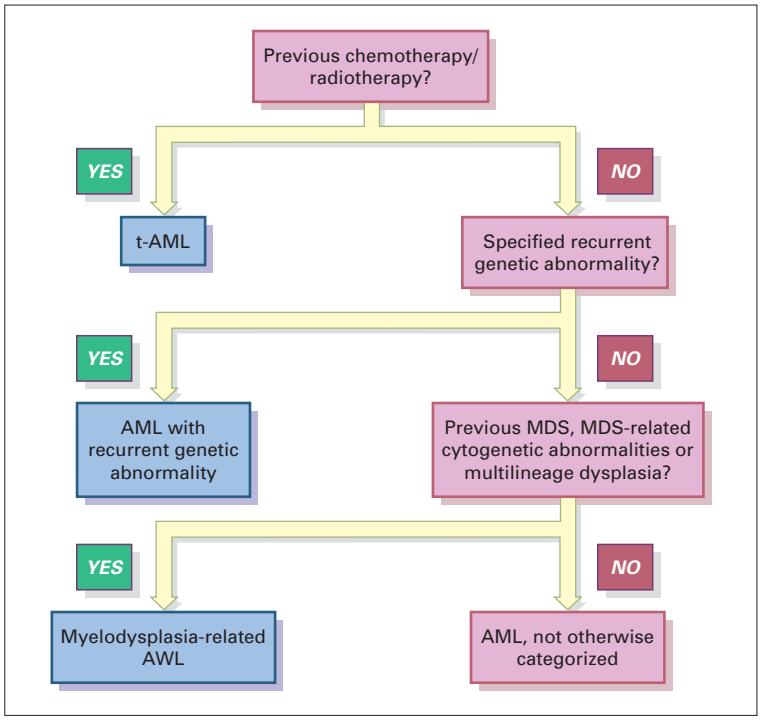


Fig. 3.1 Algorithm showing how the World Health Organization (WHO) 2008 classification of acute myeloid leukaemia (AML) is applied. MDS, myelodysplastic syndrome.

characteristics. When cases cannot be assigned to recognizable cytogenetic/genetic categories, the classification becomes phenotypic.

The WHO classification of acute myeloid leukaemia

The WHO criteria for regarding a patient as having acute myeloid leukaemia (AML) differ from the FAB criteria in that cases with at least 20% bone marrow or peripheral blood blasts are categorized as AML rather than as a myelodysplastic syndrome (MDS) whereas in the FAB classification the threshold was 30% and applied only to the bone marrow; in the WHO classification the blast count also includes promonocytes.

The WHO classification of AML is summarized in Table 3.1 [5]. Classification is hierarchical, as shown in Fig. 3.1, with therapy-related cases being assigned first, then cases with certain specified recurrent genetic abnormalities, followed by cases with myelodysplasia-related features. Finally, the remaining cases are assigned on the basis of cytological features to categories that have many similarities to the FAB categories of AML but differ from them and also include several additional entities. In addition, myeloid neoplasms associated with Down syndrome and blastic plasmacytoid dendritic cell neoplasm are assigned to two separate specific categories.

Acute myeloid leukaemia with recurrent genetic abnormalities

In the 2008 WHO classification 'AML with recurrent genetic abnormalities' has been expanded by the inclusion of three new categories with recurrent cytogenetic/genetic abnormalities, specifically t(6;9)(p23;q34), inv(3)(q21q26.2)/t(3;3)(q21;q26.2) and t(1;22)(p13;q13) [6]. In addition, two provisional categories have been introduced that have a recurrent genetic abnormality without any specific associated cytogenetic abnormality. There are other recurrent genetic abnormalities that should be noted because of their prognostic significance although they do not constitute separate categories, in part because they overlap with other well-defined genetic categories.

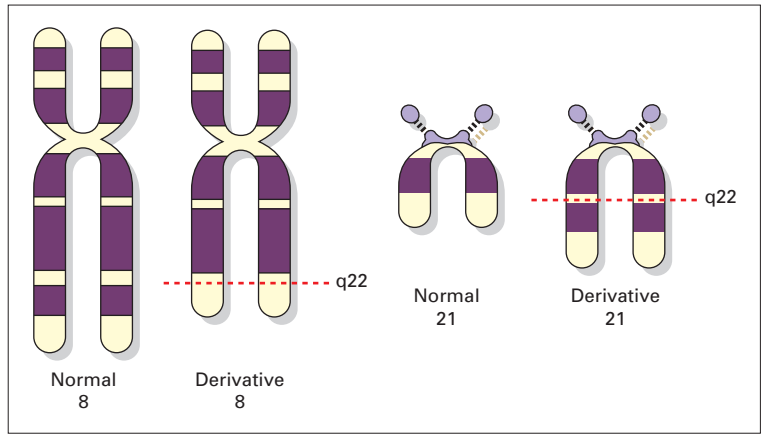


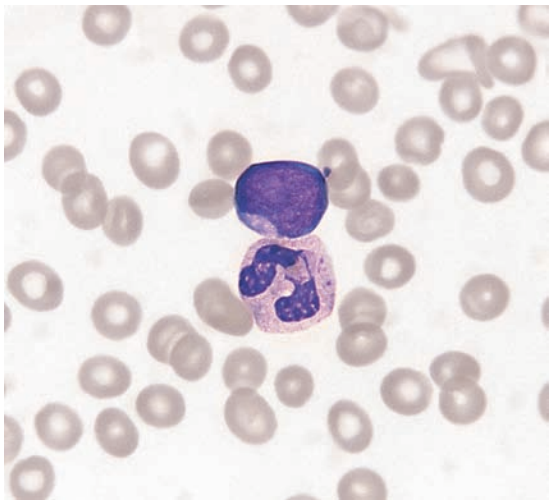
Fig. 3.2 A diagrammatic representation of the t(8;21)(q22;q22) abnormality. The breakpoints in the two derivative chromosomes are indicated. (Modified from reference 2.)

Acute myeloid leukaemia with t(8;21)(q22;q22); RUNX1-RUNX1T1

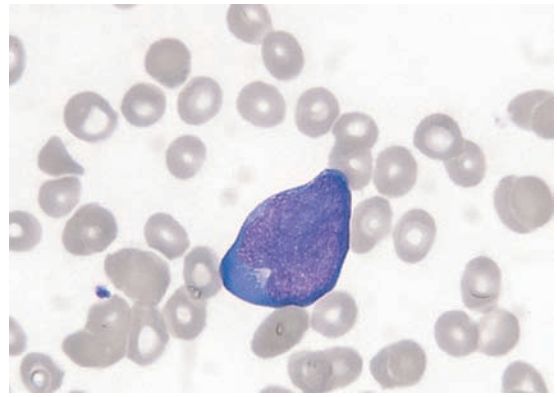
t(8;21)(q22;q22) [7–12] (Fig. 3.2), discovered by Rowley in 1972 [7] was the first balanced translocation recognized in AML. It is associated with a *RUNX1-RUNX1T1* fusion gene (previously known as *AML1-ETO*). It is one of the two most common specific translocations found, the other being t(15;17). Overall, such cases comprise 4–9% of AML in different series of patients [13–15]; in a series of 1897 unselected adult patients prevalence was 4.6% [16]. The frequency is higher in children (12–14% of AML) than in adults (6% of AML) [14,17]. In elderly adults, the prevalence falls to 2% [18]. Adult patients are usually young and more often male than female. In around half of childhood cases the translocation appears to have occurred *in utero* [19]; a second mutation occurring in extra-uterine life may be necessary for the leukaemic phenotype. The geographical distribution appears to be uneven with a higher percentage of AML patients with this abnormality being found in Japan, among non-whites in South Africa [10] and in China [20]. The prevalence of t(8;21) is similar among *de novo* and therapy-related cases [16]. There appears to be a more than coincidental association between this subtype of AML and systemic mastocytosis [21].

Clinical and haematological features t(8;21) is strongly associated with the FAB category of M2 AML, with a minority of cases being M1 or, less often, M4. The great majority of cases are *de novo* but abnormalities of 21q22, including t(8;21)(q22;q22), are found among cases of therapy-related AML (t-AML) following exposure to topoisomerase II-interactive drugs including etoposide and the anthracyclines [22,23]; in the WHO classification, such cases, which have a 5-year survival of only 30% [24], are categorized as t-AML not as AML with a recurrent genetic abnormality. This subtype of leukaemia may also be linked to prior exposure to benzene [24]. Formation of myeloid sarcomas, solid tumours of leukaemic cells, is not uncommon [25]. The complete remission rate and median survival are relatively favourable with a complete remission rate of around 90% and a 5-year survival of 50% or more [18,26–28]. A series of 401 patients entered into Medical Research Council (MRC) trials with a long median follow-up showed a 10-year survival of 61% [29]. Prognosis is good in younger patients but intermediate in older patients [30]. Prognosis is significantly better in those with a presenting white blood cell count (WBC) of 20×10^9/l or less [31]. Worse prognosis is also indicated by a higher value for the product of the WBC and the bone marrow blast percentage, with cut-off points of 2.5 and 20 dividing patients into three prognostic groups [32].

Characteristic cytological features are observed [11,12,33,34] (Figs 3.3–3.7); see also Fig. 1.2). There is maturation of leukaemic cells to neutrophils and consequently severe neutropenia is uncommon. Some patients have neutrophilia. The blasts are very heterogeneous, variable in size but often large and with a high nucleocytoplasmic ratio. Nuclei are commonly indented or cleft with large



(a)

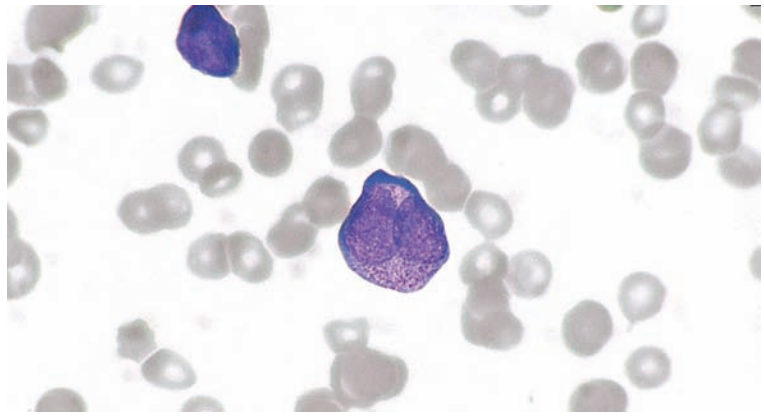


(b)

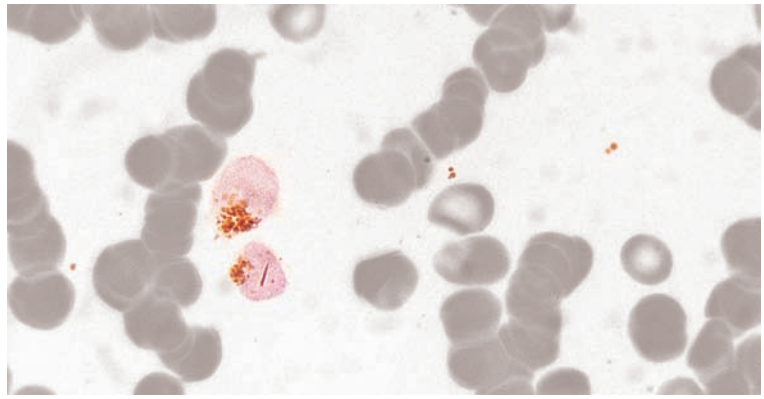
Fig. 3.3 Peripheral blood (PB) and bone marrow (BM) films of a patient with AML associated with t(8;21)(q22;q22), French–American–British (FAB) M2 AML. (a) PB film showing a blast cell and an abnormal neutrophil. May–Grünwald–Giemsa (MGG) ×100. (b) PB film showing a blast cell with strongly basophilic cytoplasm and a paranuclear hof representing the Golgi zone. MGG ×100.

nucleoli. Cytoplasm may be basophilic (Fig. 3.3b), sometimes vacuolated. Basophilia is sometimes confined to the periphery of the cytoplasm (Fig. 3.3c). Auer rods are common with often a single, slender Auer rod per cell. The Auer rods may be fusiform. Some blasts may contain giant granules as may maturing cells. In individual cases, blasts may contain Auer rods, giant granules, both or neither. Binucleated myeloblasts, promyelocytes, myelocytes and metamyelocytes are seen [8]. Neutrophils often show hypogranularity, bizarre-shaped nuclei and the acquired Pelger–Huët anomaly. Homogeneous salmon-pink cytoplasm of mature neutrophils is characteristic [34]. Maturing granulocytes may contain Auer rods, which are sometimes even found in metamyelocytes and neutrophils (Fig. 3.6a). Auer rods may also be observed in macrophages (Fig. 3.6b). An unusual feature, striking haemophagocytosis by neutrophils, has been described in one patient [35]. Bone marrow eosinophilia occurs in a proportion of patients (Fig. 3.7). Eosinophil granules vary in their staining characteristics, appearing orange, green/grey or blue [36]; although some may have granules which are basophilic this feature is much less marked than in inv(16)/M4Eo (see below) [9]. Most patients do not have a peripheral blood eosinophilia but occasional patients have had a markedly elevated eosinophil count [37,38] sometimes with an associated hypereosinophilic syndrome [38]. An increase of bone marrow basophils or mast cells occurs in a significant proportion of cases [33] and sometimes this is striking [39]. The mast cells have been demonstrated, in one patient, to be part of the neoplastic clone [40]. Rarely the presentation is as acute basophilic leukaemia [41,42]. Although maturing granulocytes usually show dysplasia, other lineages usually do not; myelodysplastic features in erythroid and megakaryocyte lineages have been reported as uncommon in some series [12] and not present in others [11,34]. There may be a discrepancy in blast numbers between the bone marrow and the blood. Occasional cases present with bone marrow blast cells below 20%. If untreated, cases with low blast cell counts evolve rapidly into overt AML [43] and their recognition as AML in the WHO classification is clearly appropriate. Therapy-related AML with t(8;21) differs in that there may be a preceding myelodysplastic phase and trilineage myelodysplasia.

Cytochemical stains [11,44] show localized Sudan black B (SBB) and myeloperoxidase (MPO) positivity in the blasts, often confined to the cleft or hof of the nucleus (Fig. 3.3d). Chloroacetate esterase (CAE) is strongly positive. There may also



(c)



(d)

Fig. 3.3 (*Continued*) (c) PB film showing an abnormal promyelocyte with peripheral basophilia. MGG ×100. (d) BM film showing two blasts with peroxidase-positive granules in the hof of the nucleus. One blast also contains an Auer rod. Peroxidase ×100.

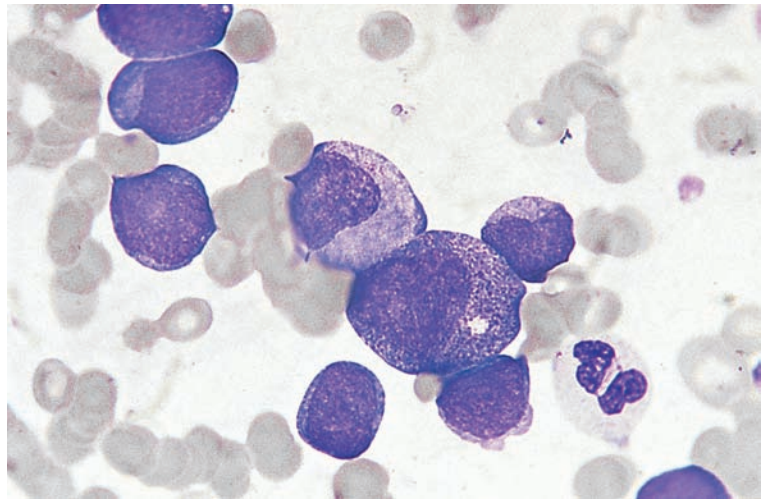


Fig. 3.4 BM film from a patient with AML associated with t(8;21)(q22;q22), FAB M2 AML, showing blasts and maturing granulocytic cells including a hypogranular neutrophil. MGG ×100.

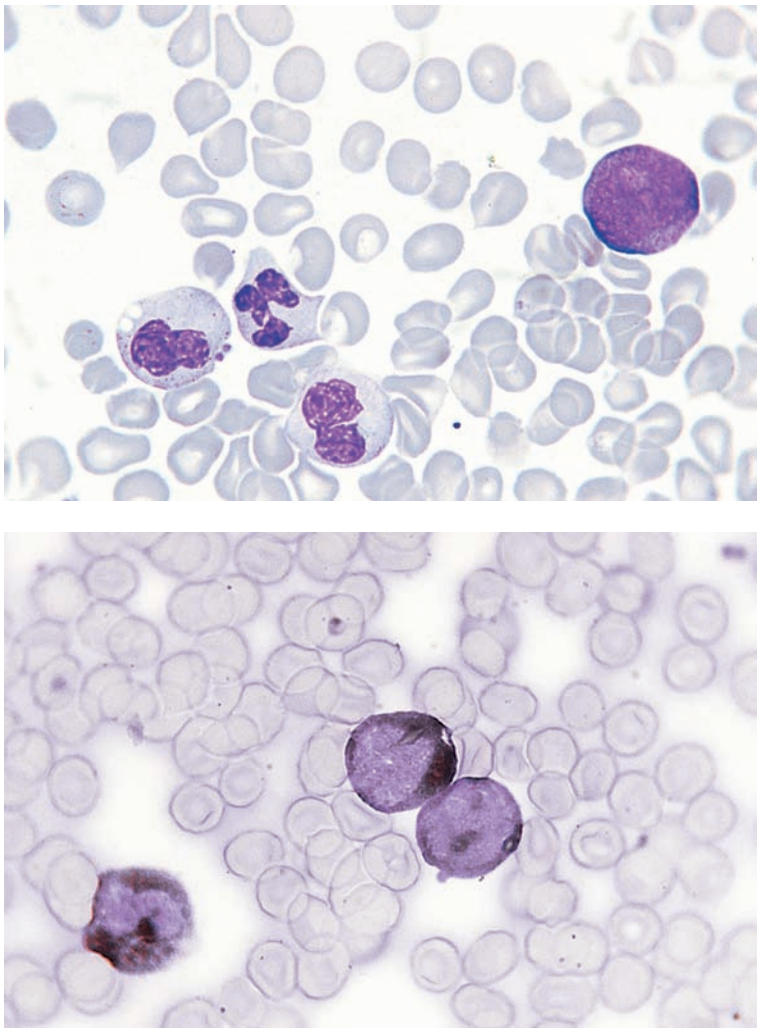
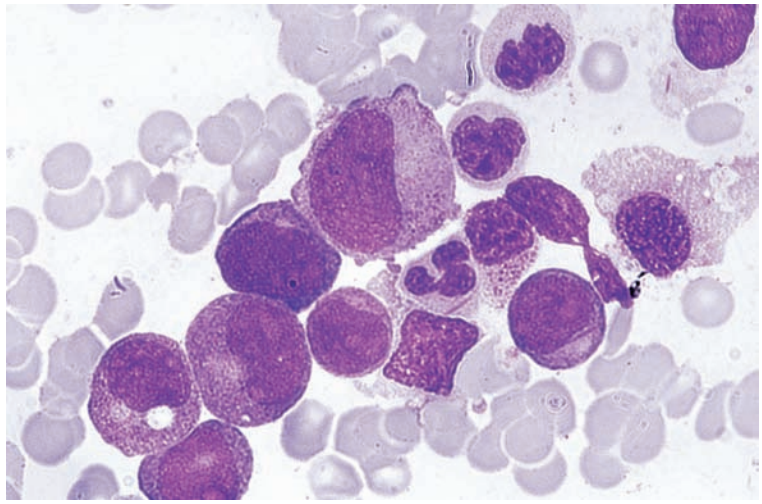


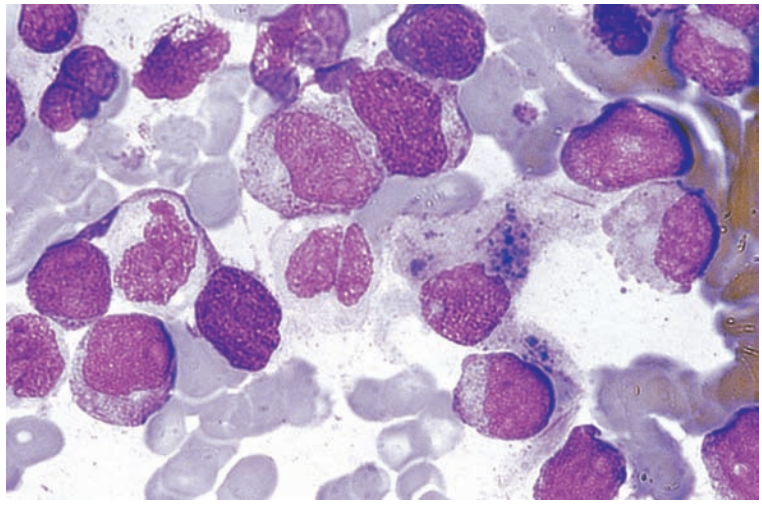
Fig. 3.5 BM film of a patient with AML associated with t(8;21)(q22;q22), FAB M2 AML. (a) A blast and three abnormal neutrophils. MGG ×100. (b) Sudan black B (SBB) stain showing strongly positive cells in one of which an Auer rod with a hollow core can be seen. SBB ×100.

be Golgi zone positivity for α-naphthyl acetate esterase [12]. An MPO reaction may show Auer rods to be multiple and occasionally they are revealed in eosinophils as well as in the neutrophil series; Auer rods are sometimes positive for CAE and periodic acid–Schiff (PAS) as well as for MPO and SBB. Auer rods may have a non-staining core (Fig. 3.5b). The eosinophils in AML associated with t(8;21) do not show the aberrant positivity for CAE, which is a feature of eosinophils in AML associated with inv(16) [9]. The neutrophil alkaline phosphatase score is generally low [45] but neutrophils that are negative for SBB and MPO are uncommon [44]. Blasts are more commonly PAS positive than in AML in general; the pattern of staining is diffuse with some granules and rare blocks. PAS-positive erythroblasts are not a feature. Eosinophil granules may show aberrant PAS positivity but this is less a feature than for AML with inv(16) [11] (see below).

This category of AML has a relatively good prognosis, with 5-year survivals in adults of up to 70% (but usually *c.* 50%) being reported with chemotherapy alone [46,47]. For this reason, stem cell transplantation in first remission is considered not to be indicated. In one large series, survival was no better with stem cell transplantation [32]. Intensive treatment with high dose cytarabine



(a)



(b)

Fig. 3.6 BM film from a patient with AML associated with t(8;21)(q22;q22), FAB M2 AML. (a) A spectrum of maturing cells of granulocyte lineage – a blast cell and a neutrophil contain long thin Auer rods. (b) A macrophage containing an Auer rod. MGG ×100.

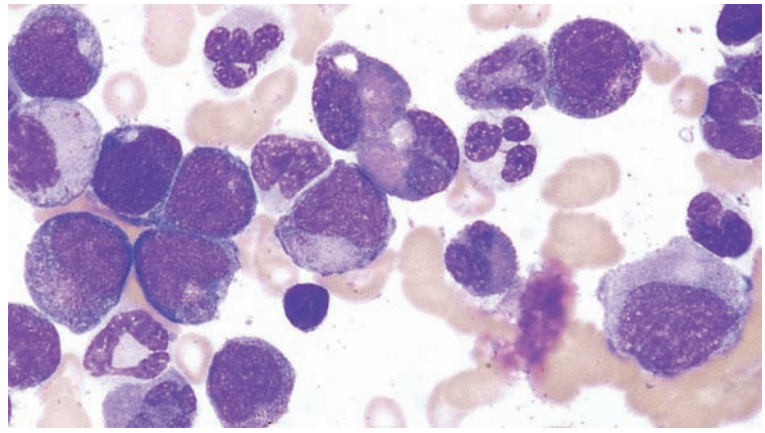


Fig. 3.7 BM film of a patient with t(8;21)(q22;q22), FAB M2 AML with eosinophilia, showing maturing cells of neutrophil and eosinophil lineage; there are three eosinophil myelocytes and two mature eosinophils. MGG ×100.

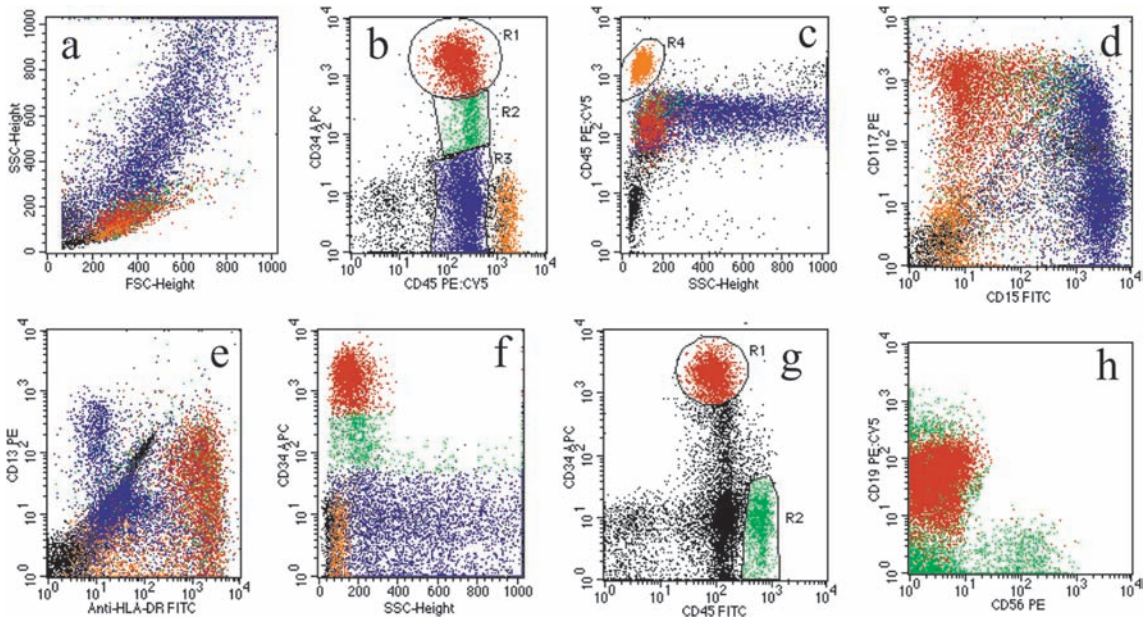


Fig. 3.8 Four-colour flow cytometry immunophenotyping in a patient with AML associated with t(8;21)(q22;q22). Plots a, b and c show standard analysis regions set to delineate myeloid blast differentiation (compare with Fig. 2.3); plots d and e show CD117/CD15 and CD13/HLA-DR expression by these individual populations. The most immature blasts cells (R1) are CD117+ CD15– CD13+ HLA-DR+. Maturing blasts (R2) are CD117+ CD15+ and mature myeloid cells (R3) are CD15+ CD117+/– HLA-DR weak. Plots g and h show CD34 expression and CD19 expression by blasts cells, though at levels weaker than on normal B cells. (By courtesy of Dr Steve Richards, Leeds.)

appears to be important in achieving long-term survival [46]. The prognosis in children appears similarly good [47].

This translocation retains a relatively good prognostic significance in patients in first relapse and is incorporated into the European Prognostic Index [48].

Immunophenotype This category of AML has a characteristic immunophenotype [49] (Fig. 3.8). Blast cells express CD34, human leucocyte antigen DR (HLA-DR), CD117 and MPO in more than 95% of cases [50]. They are also typically positive for CD13 (75%), CD33 (>85%) and CD65 [50]. Rare cases are negative for CD13, CD33 and CD14 but are positive for MPO [51,52]. Expression of CD34, HLA-DR and MPO is stronger than in other cases of AML whereas expression of CD13 and CD33 is more likely to be absent or weak [53]. Expression of CD13 is usually, however, stronger than expression of CD33 [54]. CD11b and CD15 are expressed mainly on the maturing granulocytic cells [55] but aberrant coexpression of CD34 and CD15 is seen in a significant minority of cases. CD11c is expressed in half or more of cases [50,56]; expression of CD11a/CD18 is low as a result of inhibition of the CD11a promoter by binding of RUNX1-RUNX1T1 rather than of RUNX1 or RUNX3 [57]. There is usually positivity for the B-lineage marker CD19 (not very strong; 75% of cases in a large series) [50] and often for the natural killer (NK) cell marker CD56 (also weak; 82% in the same series) [50,55,58,59]. Expression of CD56 correlates with a worse prognosis [60,61]. CD79a is expressed in many cases, 6 of 69 in one study [62] and 10 of 19 in another [50], and should not lead to classification as mixed lineage acute leukaemia. PAX5, characteristic of the B lineage, is often expressed; expression correlates strongly with expression of CD19 and CD79a [63]. Coexpression of CD34, HLA-DR and MPO has approaching 100% sensitivity for detection of this subtype [56]. Coexpression of CD19 and CD34,

which is uncommon in other subtypes of AML, suggests a diagnosis of AML with t(8;21) [64], with coexpression of CD19, CD34 and CD56 having about 67% sensitivity and approaching 100% positive predictive value [56]. CD2, CD4, CD7, CD14, CD64 and terminal deoxynucleotidyl transferase (TdT) have been reported to be usually negative [51,55,58] and, when TdT is positive, expression is usually weak [6]; in one study TdT expression was reported more often positive [56]. PRAME, an antigen expressed in various non-haematological neoplasms, is usually expressed in AML associated with t(8;21) but is much less often expressed in other types of AML [65]. The B-cell-associated transcription factor, OCT2, may be expressed [66]. Expression of CD56 and lack of expression of CD19 has been found to be suggestive of cases with a *KIT* mutation [50].

Cytogenetic and molecular genetic features The t(8;21)(q22;q22) rearrangement, which is shown diagrammatically in Fig. 3.2, is shown in a karyogram in Fig. 3.9. Patients with variant translocations, such as t(1;21;8) and t(8;11;21), associated with *RUNX1-RUNX1T1* fusion, have the same disease characteristics [67]. MAC (morphology–antibody–chromosomes) techniques show the translocation in cells of granulocytic lineage but not in erythroid or megakaryocytic cells [68]. Eosinophils are also part of the neoplastic clone [9]. Common secondary karyotypic abnormalities are loss of the Y chromosome in males, loss of the inactive X chromosome in females and del(9q). Trisomy 8 occurs in a lower proportion of patients. The presence of secondary cytogenetic abnormalities, even complex ones, did not worsen the prognosis in one series of patients [27] but, in another series of patients who had either t(8;21) or inv(16), survival was worse in those with complex cytogenetic abnormalities [28]. In one study, loss of a sex chromosome was found to be associated with a worse prognosis [69] and in another –Y was associated with a better prognosis but neither of these observations was confirmed in other studies [47]. A small study suggested that del(9q) worsened the prognosis but this was not substantiated in a larger study [70]. A study of 401 patients entered into MRC trials with a long median follow-up found additional cytogenetic abnormalities to have no impact on survival [29].

The molecular mechanism of leukaemogenesis is fusion of part of the *RUNX1* gene at 8q22 with part of the *RUNX1T1* gene from 21q22 [71]. The normal *RUNX1* gene encodes one chain of a heterodimeric transcription factor (core binding factor, CBF) while the normal *RUNX1T1* gene is a putative transcription factor gene normally expressed in the brain. The *RUNX1-RUNX1T1* fusion gene, which is formed on the derivative chromosome 8 as a result of the translocation, encodes a chimeric protein that is expressed in the leukaemic cells. The RUNX1-RUNX1T1 protein may exert its oncogenic effect by interfering with the transcription factor activity of normal RUNX1 protein. Using microarray analysis, AML associated with t(8;21) can be distinguished from AML with t(15;17) or inv(16)/t(16;16) [72].

FLT3 mutations are considerably less common than in AML in general [73,74]; in one large study *FLT3* internal tandem duplication (*FLT3*-ITD) was

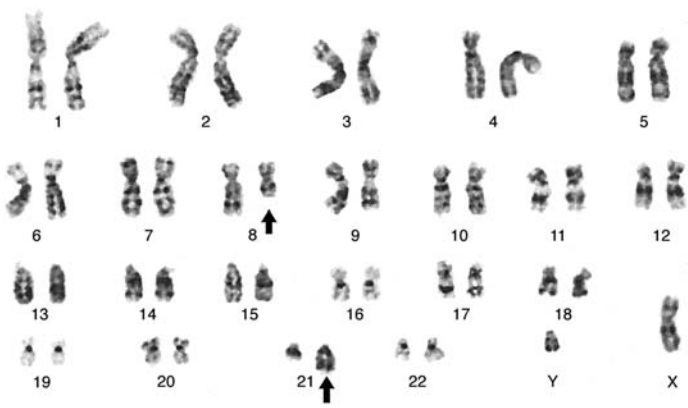


Fig. 3.9 Karyogram showing the translocation between chromosome 8 and chromosome 21 in a male patient with FAB M2 AML and the karyotype t(8;21)(q22;q22). (By courtesy of Professor Lorna Secker-Walker, London.)

found in 15% and *FLT3* tyrosine kinase domain mutation (FLT3-TKD) in 7% [75]. Mutations of *KIT* occur in a significant minority of patients – reported in 10% [76], 17% [74] and 45% (including 28% D816 mutations) [77]. Mutations, or at least *KIT* D816 mutations, are associated with a higher WBC [77], more extramedullary disease [77], a greater risk of relapse [74,77,78] and a worse survival [74,76,77]. These are gain of function mutations in codon 816 (D816V, D816H or D816Y) affecting the extracellular domain, which increase proliferation and convey resistance to apoptosis [79]. Some of the patients with a *KIT* mutation have aberrant bone marrow mast cells [76,80]. *KIT* mutations are also associated with other translocations that lead to rearrangement of the *RUNX1* gene, e.g. t(3;21), t(12;21) and t(17;21) [76]. *RAS* mutations were found to be more common than in AML in general in one study [81] but not in two others [82,83]. Mutations can be in *KRAS* or *NRAS* and do not influence prognosis [83].

t(8;21) can be detected using dual-colour, dual-fusion fluorescence *in situ* hybridization (FISH) and probes for *RUNX1* and *RUNX1T1* [84] (Figs 3.10 and 3.11). The rearrangement can also be detected

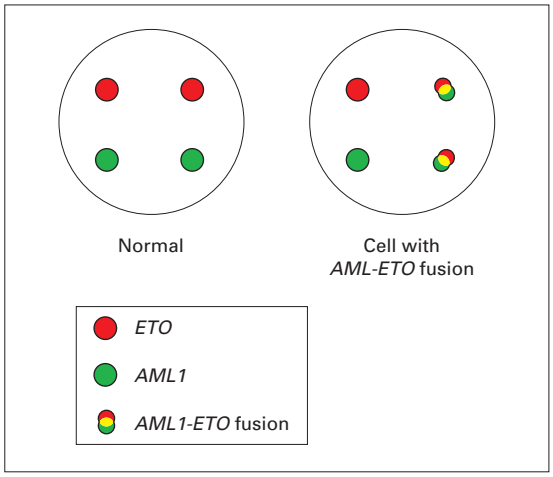


Fig. 3.10 Diagrammatic representation of dual-colour, dual-fusion fluorescence *in situ* hybridization (FISH) for the detection of *RUNX1-RUNX1T1* (*AML1-ETO*). The normal cell has two red *RUNX1T1* (*ETO*) signals and two green *RUNX1* (*AML1*) signals. The cell with a t(8;21) translocation has a normal green *RUNX1* signal, a normal red *RUNX1T1* signal and two yellow fusion signals representing *RUNX1-RUNX1T1* and *RUNX1T1-RUNX1*.

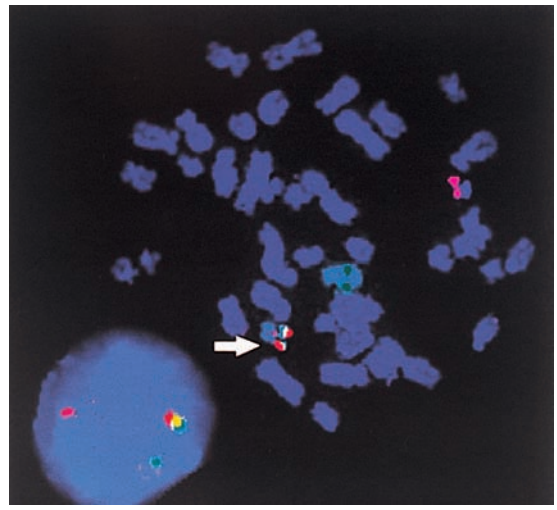


Fig. 3.11 Metaphase spread and interphase nucleus from a patient with FAB M2 AML and t(8;21)(q22;q22) hybridized with probes to the genes *RUNX1T1* (*ETO*) (green) and *RUNX1* (*AML1*) (red). These are located at 8q22 and 21q22, respectively. Note one green signal (on the normal 8), one red signal (on the normal 21) and one yellow signal (on the derived chromosome 8) indicating the fusion of the two genes resulting from the translocation. (By courtesy of Dr Christine Harrison, Newcastle.)

by reverse transcriptase polymerase chain reaction (RT-PCR) in cytologically typical cases both with and without t(8;21). The cases with *RUNX1-RUNX1T1* but without t(8;21) may be cytogenetically normal or have complex chromosomal rearrangements (involving chromosomes 8 and 21 together with a third chromosome) or deletions or other abnormalities of chromosome 8 [33,85]. Use of molecular techniques has been reported to increase the number of cases identified by up to 60% in comparison with cytogenetic analysis alone [86], but in another large study only 2 of 33 cases (6%) were not detected by cytogenetic analysis [15].

The detection of minimal residual disease (MRD) does not necessarily presage haematological relapse. However, when MRD is monitored by by real-time quantitative PCR (RQ-PCR), the quantity of fusion gene transcript present at presentation and after consolidation chemotherapy is predictive for event-free survival [87]. A 1 log or greater increase in transcript from the remission level is predictive of relapse [88].

Acute myeloid leukaemia with other RUNX1 rearrangement

Acute myeloid leukaemia associated with t(16;21)(q24;q22) and *RUNX1-CBFA2T3* (previously known as *AML1-MTG16*) (see Table 3.7) appears to be closely related to AML with t(8;21), with a similar molecular mechanism, FAB M2 morphology with or without eosinophilia and possible coexpression of CD19 and CD34 [89]. However cases with t(16;21) are often therapy related and there may be preceding MDS. Trisomy 8 is a frequent secondary abnormality [90]. FISH – using probes designed to detect t(8;21) or t(12;21) – can be used to demonstrate rearrangement of *RUNX1*.

Acute promyelocytic leukaemia with t(15;17)(q22;q12); PML-RARA

t(15;17)(q22;q12), previously identified as t(15;17)(q22;q21) (Fig. 3.12), is present in the great majority of patients with acute promyelocytic leukaemia (FAB types M3 and M3V AML) and, with the exception of rare conditions such as hypergranular promyelocytic transformation of either chronic granulocytic leukaemia or polycythaemia vera, is confined to this category of leukaemia. This subtype of AML was clearly described by Hillestad in 1957 but he considered the first reported case to have been that of Risak in 1935 [91,92]. The microgranular variant was described in 1980 [93] and the hyperbasophilic microgranular variant in 1982 [94]. An abnormal chromosome 17 was first reported in 1976 [95] and in 1977 the same group recognized the reciprocal translocation between chromosomes 15 and 17 [96]. Overall, this subtype constitutes 4–9% of cases of AML in different series of patients [13,14]; in a series of 1897 unselected adult patients prevalence was 5.2% [16]. Among cases of AML in elderly adults, the prevalence falls to 4% [18]. This type of leukaemia appears to be more common in Costa Rica, Nicaragua and Venezuela and in Mexican Mestizos and the Hispanic population in the USA [97]. It may also be more common in Spaniards than in other Europeans. Cases in children may have an intrauterine origin with a latency of about 10 years [98]. Prevalence does not differ significantly between *de novo* and therapy-related cases of AML [16] and in one series of patients 10% of cases of acute promyelocytic leukaemia were therapy related [99], following topoisomerase II-interactive drugs and other agents [22,23,100]. The epidermal growth factor inhibitor, gefitinib, used to treat carcinoma of the lung appears to be another potential cause of this subtype of AML [101]. Therapy-related cases, which are classified as such rather than as 'AML with recurrent genetic abnormality', have generally followed anthracyclines and mitoxantrone rather than etoposide. There may also be an increased incidence in women with previous breast cancer, regardless of whether or not chemotherapy was administered.

Clinical and haematological features Disseminated intravascular coagulation with markedly increased fibrinolysis is common and patients often present with haemorrhage. The distinctive cytological and cytochemical features have been described on page 21. Presentation with extramedullary disease is rare [102]. The platelet count tends to be lower than in

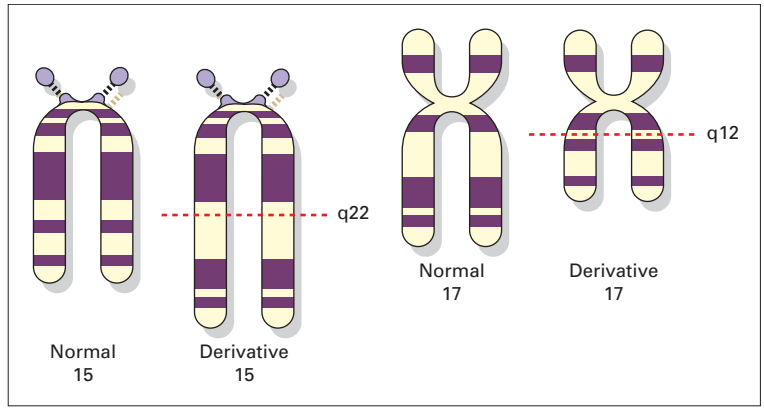


Fig. 3.12 A diagrammatic representation of the t(15;17)(q22;q12) abnormality. (Modified from reference 2.)

other types of acute leukaemia. The WBC is often normal or low in hypergranular promyelocytic leukaemia but usually elevated in the microgranular/hypogranular variant. An uncommon manifestation is haemolytic uraemic syndrome with schistocytes in the blood film and thrombotic microangiopathy affecting the kidneys [103]. Although *de novo* acute promyelocytic leukaemia does not show dysplasia of other lineages, both erythroid and megakaryocytic dysplasia are quite common in t-AML with the same translocation [99].

Historically, the complete remission rate and median survival were relatively favourable [18,26,27] and in recent years these has improved further. This subtype of AML has a particular sensitivity to differentiation therapy with all-*trans*-retinoic acid (ATRA, tretinoin), a discovery first made in China by Zhen-Yi Wang and colleagues [104], who treated their first patient in 1985. With modern treatment incorporating this agent, the prognosis is good with reported remission rates as high as 85–90% and 5-year survivals of 60–80% [47,105, 106] or higher. A study of 759 patients entered into MRC trials showed a 10-year survival of 73% [29]. Five-year survival in t-AML with this translocation approaches 50% and is thus considerably better than in most other types of t-AML [100]. In a study comparing 'secondary' and *de novo* cases treated according to the same protocols, responses to treatment and survival were identical in the two groups [107]. However, it should be noted not all the 'secondary' cases in this series are likely to have been therapy related; only half the patients had received chemotherapy and more than a quarter had been treated for a first neoplasm by surgery alone [107]. Prognosis is best in those with a lower presenting WBC (less than 2, 5 or 10×10^9/l in different trials [106,108–110]) and a presenting platelet count greater than 40×10^9/l [106,111]; the worse prognosis in those with a higher WBC is attributable to a higher early death rate. Prognosis has been found to be worse in the hypogranular/microgranular variant than in hypergranular promyelocytic leukaemia in some series of patients [110] but not in others [112], but in view of the higher WBC that characterizes the microgranular variant it is likely that there is a genuine difference in prognosis.

In addition to ATRA, arsenic trioxide (As_2O_3) and tetra-arsenic tetra-sulphide (As_4S_4) are efficacious [113], these therapies also having been pioneered in China from 1992 onwards [114]. Among chemotherapeutic agents, anthracyclines (either daunorubicin or idarubicin) are of considerable importance, whereas cytarabine may be of little or no importance [108,115]. Anthracyclines as single agents are associated with complete remission rates of 60–80% and very high survival rates are achieved with anthracyclines and ATRA alone [105]. CD33-targeted monoclonal antibodies (gemtuzumab ozagamicin, 'Myelotarg') may also contribute to successful outcome [116].

Both ATRA and arsenic trioxide therapy lead to maturation of cells of the leukaemic clone with sometimes a steep rise in the WBC and with the appearance of maturing but cytologically abnormal cells. In addition, arsenic trioxide can trigger apoptosis [114]. The two drugs are synergistic. Tetra-arsenic tetra-sulphide differs in that it leads to apoptosis rather than differentiation of promyelocytes [113].

Immunophenotype This category of AML has a characteristic immunophenotype [49] (Fig. 3.13), which can be useful in distinguishing it from other subtypes of AML, e.g. in distinguishing the hypogranular variant from acute myelomonocytic leukaemia. The immunophenotype of the hypogranular variant was found to be less distinctive than that of hypergranular cases in several studies [117,118] whereas in another large study no difference was detected [119]. Flow cytometry shows, on light scatter measurements, a compact cluster with relatively high forward and sideways light scatter [120]. However, high sideways scatter, attributable to the granularity of the cells, is not invariably increased and is less likely to be increased in the variant form [121]. There may be significant autofluorescence [122] so that reactions can only be regarded as positive if they are above that of an autologous control. CD13, CD33 and MPO are characteristically positive but CD33 is more consistently positive than CD13 [117]. CD33 expression is homogeneous [49], whereas CD13 expression tends to be heterogeneous [49,119,123]. CD33 is characteristically more strongly expressed than in normal neutrophils [122]. HLA-DR and CD34, which are positive on early granulocytic lineage cells but negative on normal promyelocytes, are

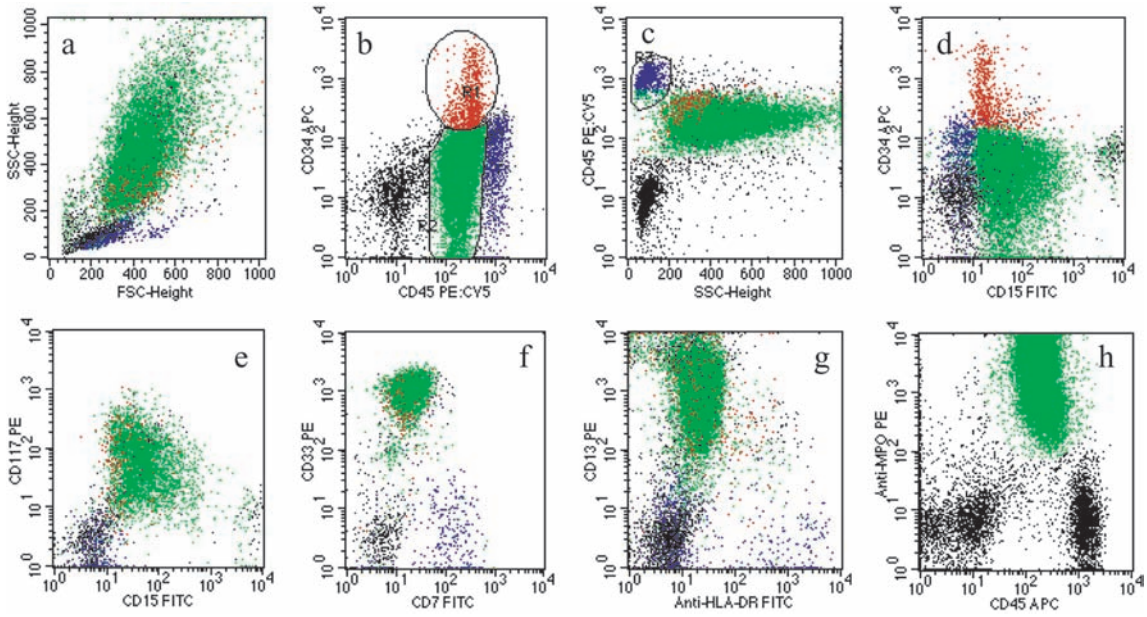


Fig. 3.13 Four-colour flow cytometry immunophenotyping from a patient with AML associated with t(15;17) (compare with Fig. 2.3). Plots a, b and c show standard analysis regions. The blast cells in this subtype of AML are usually CD34– CD45+. Their key phenotypic features are shown in plots d–h. In plot d the blast cells have clearly lost CD34 expression but, in contrast to normal myeloid cells at this stage of differentiation, have not acquired CD15. Note the strong homogeneous CD33 expression (f), heterogeneous CD13 expression covering 2 log decades of fluorescence (g), the absence of HLA-DR expression (g) and the strong myeloperoxidase expression (h). (By courtesy of Dr Steve Richards.)

usually negative but expression is variable [122] and approaching a fifth of cases may be positive for CD34, particularly in the hypogranular variant [112,117,118,124–126]; CD34 expression on individual cells may range from negative to positive. In one study about 80% of cases were negative for both CD34 and HLA-DR in comparison with about 10% of other cases of AML [127]. CD34 expression has been found to be an independent poor prognostic factor, as well as correlating with variant cytology and a higher WBC [126]. CD105, CD109 and CD133, other markers of immaturity, are negative [121,128]. TdT is positive in a minority of patients [119]. CD38 may be expressed but expression is low [121]. CD45 expression is usually weak, with stronger expression being more likely in the variant form [121]. Positive reactions for CD11b and CD14 are seen in a minority of cases; these reactions, more typical of monocytes, do not correlate with anomalous expression of non-specific esterase [129]. CD64, which is also characteristic of the monocytic lineage, is usually positive whereas CD65 has been reported to be more often negative [51] and to be weaker in the variant form [121]. A CD65 antibody recognizing CD65s gives stronger reactions than CD65 antibodies that recognize the asialo form on the antigen [121]. Variation between antibodies is the likely explanation of the inconsistent expression of CD65 sometimes reported [123]. CD117 has been reported to be more often negative [51] but others have found CD117 to be sometimes [117,118] or often [119] positive or consistently positive but with variable strength of expression [121,123]. CD15, which is expressed by normal promyelocytes, has been reported as negative [117,123] or often positive [118]; these conflicting reports may relate to the observation that antibodies recognizing sialyated CD15 usually give positive results whereas those recognizing the asialo form are negative [121]. CD11a, which is often expressed

in other categories of AML (but not in t(8;21)-associated AML), is not expressed [121,130]. CD18 is usually weak or absent and the immunophenotype of negative or weak HLA-DR, CD11a and CD18 has been found to be most predictive of a diagnosis of acute promyelocytic leukaemia [121]. HLA-DR is, however, more often positive in the variant form [112]. CD56 is usually negative [124] and when expressed (in about 20% of patients) has been correlated with extramedullary relapse [131] and with worse outcome [121,132]. CD2, CD4 and CD19 have been reported to be positive in a significant minority of cases [55,119,125] with expression of these antigens being reported to be more likely in the variant form [112,117,124]; however, others have reported that CD19 is invariably negative [121] as is CD79a [121]. In one study, CD2 expression was associated with variant morphology and with a better response rate and event-free survival [133] whereas, in two further studies, expression of CD2 was associated with variant morphology and a higher WBC [112,134] and with either a shorter duration of compete remission [112] or worse survival [134]. CD24, which is positive in normal cells of neutrophil lineage from the promyelocyte stage onwards, is negative [121]. CD16 is negative. CD7 is usually negative [135]. CD9 has been reported to be almost always positive whereas it is negative in most other subtypes of AML but not all groups have found the same sensitivity and specificity of this marker [136]. Cases of AML that are HLA-DR negative and CD2 positive are likely to be acute promyelocytic leukaemia [52]. Although there have been many reports of a difference in immunophenotype between the classical and variant forms, as detailed above, it should be noted that one quite large study (and overall 6 of 11 studies) did not find any significant differences [119].

Although there are some conflicting data, the important features of flow cytometry immunophenotyping in acute promyelocytic leukaemia can be summarized as follows. There is high forward and sideways light scatter and weak CD45 expression. CD33 and CD117 are more consistently positive than CD13. Reactions for CD34, HLA-DR, CD11a, CD11b and CD18 are most often weak or negative. CD2 may be positive but CD7 is negative. B-cell antigens are negative.

Cytogenetic and molecular genetic features The frequency with which the specific t(15;17)(q22;q12) translocation (Fig. 3.14) is detected is method dependent since direct examination without culture may result in only non-clonal erythroid cells entering mitosis [137]. MAC techniques show t(15;17) in the granulocytic lineage but not in erythroid or megakaryocyte lineages [68]. Typically, the translocation is detected by conventional cytogenetic techniques in about 90% of cases. Other patients have a *PML-RARA* fusion gene (or, less often, only an *RARA-PML* fusion gene, which may have a different significance) formed by insertion. In addition to the primary abnormality, 30–40% of cases show secondary karyotypic abnormalities. Among these the commonest are trisomy 8 (present in 12% of patients) and del(7q), del(9q) and +21 (each present in 1–2% of patients) [138]. An isochromosome of the long arm of the derivative 17, ider(17q), which results in two copies of the *PML-RARA* fusion gene, is observed in fewer than

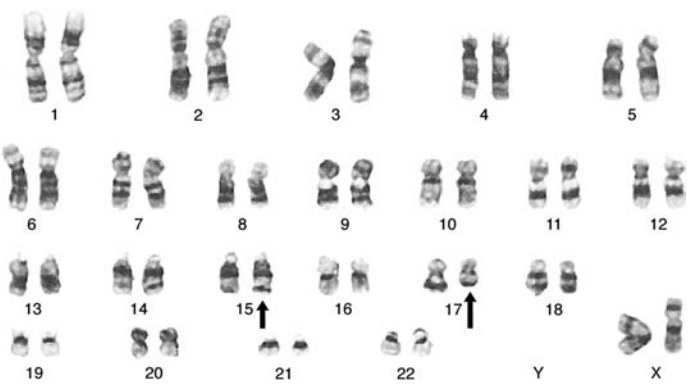


Fig. 3.14 Karyogram showing the translocation between chromosomes 15 and 17 in a female patient with FAB M3 AML and the karyotype 46,XX,t(15;17)(q22;q12). (By courtesy of Professor Lorna Secker-Walker.)

1% of patients [138]; in one study, the seven cases with ider(17)(q10)t(15;17)(q22;q12) were all hypergranular rather than variant cases [124]. An adverse prognostic significance of secondary chromosomal anomalies was observed in one series of patients [139] but not in two others [138,140]. A study of 759 patients entered into MRC trials found additional cytogenetic abnormalities to have no impact on survival [29]. Cases that lack Auer rods appear to be more likely to have additional chromosomal abnormalities [141]. Therapy-related AML with this translocation is significantly more likely to have secondary chromosomal abnormalities [99].

Classical M3 AML may also be associated with complex variant translocations, simple variant translocations and cryptic or masked translocations; these usually lead to *PML-RARA* expression but occasionally to expression only of *RARA-PML* [142]. A complex translocation involves chromosomes 15 and 17 together with a third chromosome. A simple variant translocation involves either 15 or 17 (more often 17) and another chromosome. In the case of simple variant translocations there may be a cryptic insertion in addition to the translocation. In cryptic or masked translocations both chromosomes 15 and 17 appear normal, with the karyotype either being normal or showing an unrelated abnormality; *PML-RARA* fusion results from insertion of chromosome 17 material into 15 or, less often, insertion of chromosome 15 material into 17. In all these cytogenetic variants the presence of *PML-RARA* leads to the case being assigned to this category of AML. *PML-RARA* fusion is very rare among cases of AML not recognized morphologically as M3 or M3 variant. In one study only a single instance was observed among 530 cases [143]; on morphological review, it was reclassified from M5 to M3 variant AML.

The molecular mechanism of leukaemogenesis is fusion of part of the *PML* (promyelocytic leukaemia) gene at 15q22 with part of the *RARA* (retinoic acid receptor α) gene from 17q21 [144] to form a fusion gene on the derivative chromosome 15. *PML* and *RARA* encode transcription factors. The PML-RARA fusion protein may be oncogenic because of its ability to sequester normal PML protein. In normal cells, immunofluorescence demonstrates that PML protein occurs in 10–30 discrete bodies within the nucleus (nuclear bodies), whereas in this subtype of AML there is a microparticulate or speckled distribution. Detection of this characteristic pattern by immunocytochemistry has been found to be a reliable method of diagnosis [145,146] (Fig. 3.15); the necessary antibody is commercially available. The fusion gene *RARA-PML* on chromosome 17 is expressed in about 70% of cases and may contribute to oncogenesis [142,147]. In one study, expression of *PML-RARA* alone was found to produce both the phenotype of acute promyelocytic leukaemia and ATRA sensitivity whereas the expression of *RARA-PML* alone

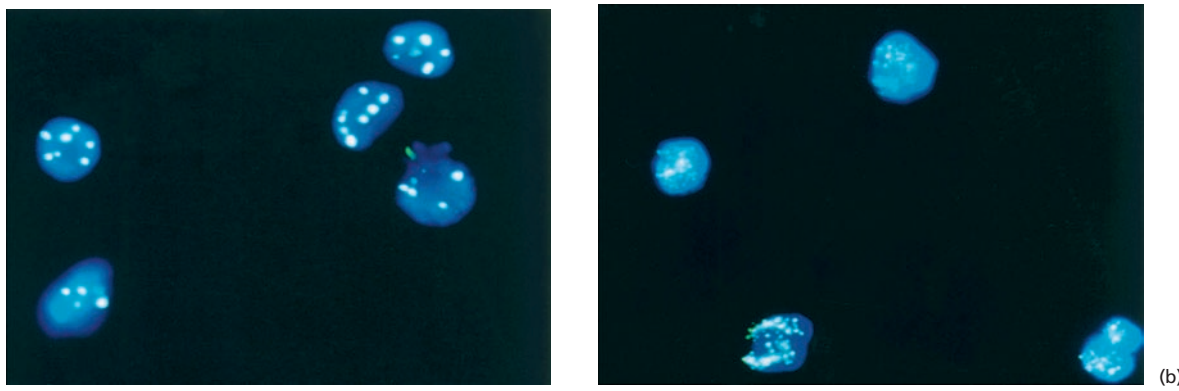


Fig. 3.15 Immunofluorescence technique using 5E10, a monoclonal antibody to promyelocytic leukaemia (PML) protein (green fluorescence); cells are counterstained with a blue fluorochrome (DAPI): (a) normal distribution of PML protein in relatively large nuclear bodies in a case of FAB M2 AML; (b) abnormal microparticulate distribution of PML protein in a case of FAB M3 AML. (By courtesy of Dr Sheila O'Connor, Leeds, and with permission of the *British Journal of Haematology* [159].)

produced the cytological features of acute promyelocytic leukaemia but without the characteristic immunostaining pattern with anti-PML antibodies or ATRA sensitivity [148]. When acute promyelocytic leukaemia occurs as a therapy-related leukaemia the precise breakpoints in the *PML* gene are clustered and differ from those in *de novo* disease [149–151].

FLT3-ITD is seen as a second genetic event in about a third of patients [152], being particularly common in those with variant cytological features [73,153–155] and correlating with a higher WBC [153–155]. In one study *FLT3*-ITD was not an independent poor prognostic feature [154] whereas in another it correlated with worse overall survival [156] and in a third with more induction deaths but no significant difference in relapse rate or overall survival [155]. *FLT3*-ITD is also a feature of therapy-related cases [99]. *FLT3*-TKD mutations occur in about 8% of cases, particularly with the variant form of the disease, and are associated with a worse prognosis, particularly if compared only with wild-type *FLT3* [83]. *NRAS* and *KRAS* mutations also occur although *NRAS* mutations are underrepresented in comparison with AML in general [157]. Hypermethylation of the *CDKN2B* promoter is associated with adverse prognosis, even after allowing for the correlation with a higher WBC [158]. Using microarray analysis, AML associated with t(15;17) can be distinguished from AML with t(8;21) or inv(16)/t(16;16) [72]. In addition, cases with classical morphology can be distinguished from the microgranular variant [72].

PML-RARA rearrangement can be detected by dual-colour, single-fusion [84,159] (Fig. 3.16a) and dual-colour, dual-fusion FISH (see Fig. 2.12). Rearrangement of *RARA* can also be detected using dual-colour, break-apart FISH (Fig. 3.16b; see also Fig. 2.11). *PML-RARA* fusion, *RARA-PML* fusion or both can be detected by RT-PCR in the great majority of patients with a cytological diagnosis of acute promyelocytic leukaemia whether or not t(15;17) is detected by karyotypic analysis. The detection rate by PCR is higher if both fusion genes are sought [160].

Detection of MRD, by RT-PCR, after consolidation therapy is of prognostic significance [108,161]. Treatment of molecular relapse appears to give superior results to waiting for haematological relapse [108]. RQ-PCR is likely to prove to be the optimal technique for molecular detection of MRD. Using this technique, the quantity of fusion gene transcript present at presentation and after consolidation therapy is predictive for event-free survival

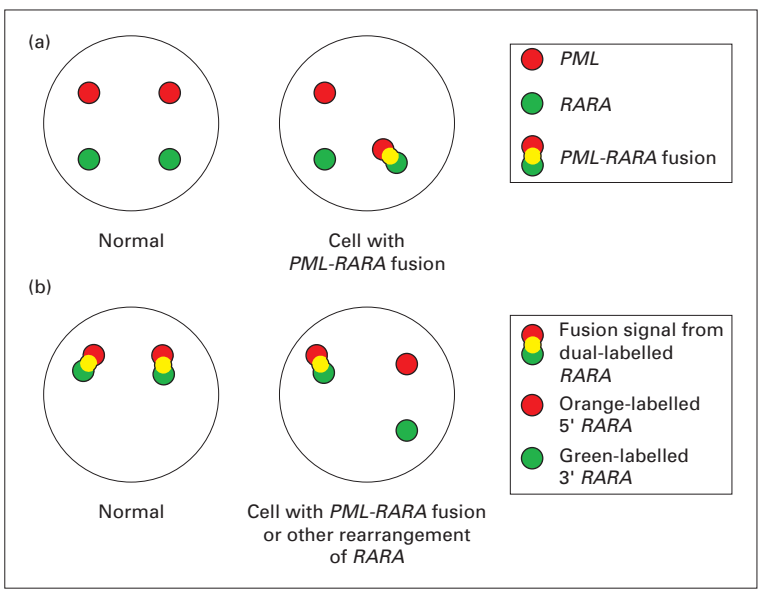


Fig. 3.16 Diagrammatic representation of two FISH strategies for the detection of *RARA* rearrangements. (a) Dual-colour, single-fusion FISH technique in t(15;17)(q22;q12). The normal cell has two red *PML* signals and two green *RARA* signals. The abnormal cell has one normal red *PML* signal, one normal green *RARA* signal and a yellow *PML-RARA* fusion signal. (b) Dual-colour, break-apart FISH technique for the detection of *RARA* disruption in t(15;17)(q22;q12) or variant translocations such as t(11;17)(q23;q21). The normal cell has two similar signals each comprised of a red signal from 5′ *RARA* and a green signal from 3′ *RARA*. The abnormal cell has a single normal red–green (yellow) signal and distinct smaller red and green signals resulting from disruption of the *RARA* gene.

[87]. Molecular monitoring by RQ-PCR during remission is necessary for optimal therapy since it permits early alteration of therapy. In patients treated by stem cell transplantation, detection of MRD is of prognostic significance and directs the need for additional therapy post-transplant [161]; however transplantation is now rarely used in this subtype of leukaemia.

Acute myeloid leukaemia with t(11;17)(q23;q21); ZBTB16-RARA or with other RARA rearrangement

In addition to t(15;17)(q22;q12), there are other translocations associated with rearrangement of the *RARA* gene with leukaemic cells often having some features reminiscent of acute promyelocytic leukaemia. However, many of these cases show cytological and clinical differences from AML with *PML-RARA* fusion. These molecular variants are summarized and compared with *PML-RARA*-related cases in Table 3.2. The most common is t(11;17)(q23;q21).

Clinical and haematological features In AML associated with t(11;17)(q23;q21); *ZBTB16-RARA* the WBC is usually not greatly elevated; the cytological features differ somewhat from those of classical hypergranular promyelocytic leukaemia, features being intermediate between those of FAB M2 and M3 categories (Fig. 3.17); multiple Auer rods and 'faggots' are not often a feature; and there may be a larger proportion of maturing granulocytes. Cells are generally more granular than those of FAB M2 AML but less granular than classical FAB M3 AML, and granules do not show the red colouration characteristic of M3 AML; giant granules are sometimes present. The nucleus is usually round rather than irregular or bilobed, there is more chromatin

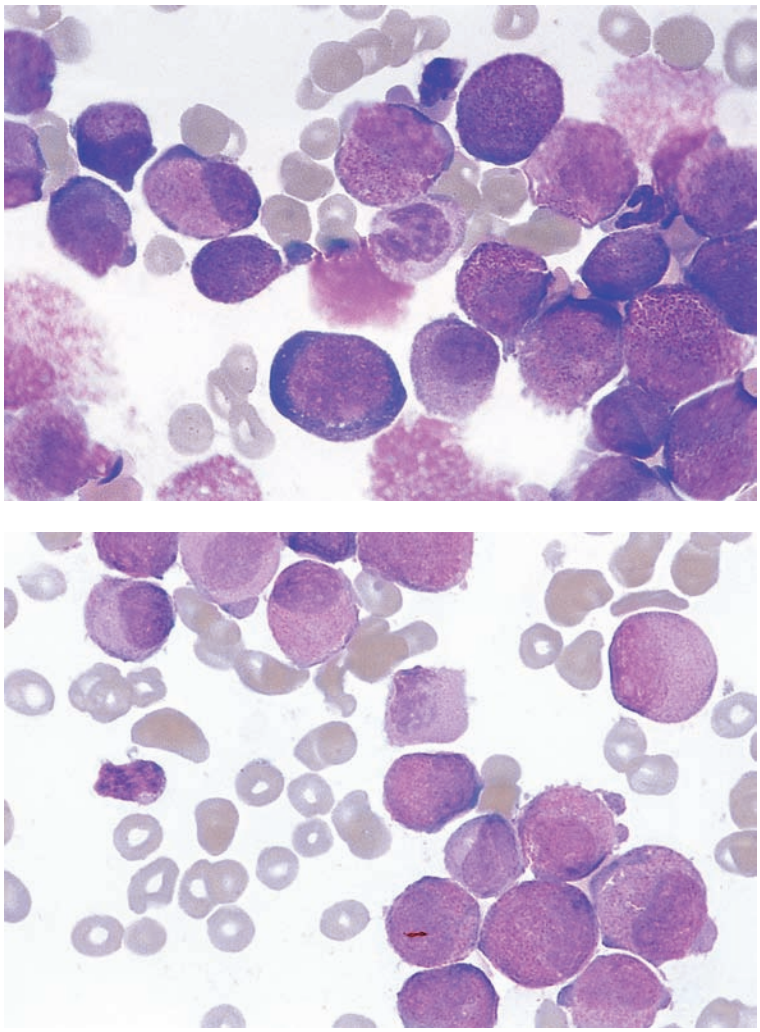
Table 3.2 Characteristics of acute myeloid leukaemia (AML) associated with rearrangement of the *RARA* gene [124,147,162–170].

Cytogenetics	Molecular genetics	Responsiveness to ATRA	Distribution of PML protein [147,165]	Frequency
t(15;17)(q22;q12)	*PML-RARA* fusion	Yes	Abnormal (i.e. microparticulate)	About 91% of FAB M3 and M3-like AML
Simple or complex variant translocations, ider(17), ins(15;17) or insertional events	*PML-RARA* fusion	Yes	Abnormal	About 7% of FAB M3 and M3-like AML
Cryptic ins(17;15)*	*RARA-PML* alone	No	Normal (i.e. discrete nuclear bodies)	
t(11;17)(q23;q12) (or normal with *RARA* insertion into 11q23)	*ZBTB16-RARA* fusion	No†	Normal (discrete nuclear bodies)	About 0.8% of FAB M3 and M3-like AML
t(11;17)(q13;q12)	*NUMA1-RARA* fusion	Probably	Normal	At least three patients
t(5;17)(q35;q12)	*NPM1-RARA* fusion	Probably	Normal	At least four patients (about 0.4% of FAB M3 and M3-like)
Interstitial deletion of chromosome 17 (one case); t(10;11)(q22;q25),i(7)(q10) (one case); –Y,+11 (one case)	*STAT5B-RARA* fusion	No (one case *in vitro* and one case *in vivo*)	Normal	At least three patients
+22 and cryptic del(17)(q21)	*PRKAR1A-RARA* fusion	Not known	Not known	One patient
t(4;17)	*FIP1L1-RARA*	Yes	Not known	One patient [168]

ATRA, all-*trans*-retinoic acid; FAB, French–American–British (classification).

* Morphologically not hypergranular promyelocytic leukaemia.

† Although lack of responsiveness is not absolute [170]; also unresponsive to arsenic trioxide therapy [166].



(a)

(b)

Fig. 3.17 BM film from a patient with FAB M2/M3 AML with t(11;17)(q23;q21) showing: (a) more cells maturing beyond promyelocyte stage than is usual in classical M3 AML but with several hypergranular blasts being present; (b) several hypergranular promyelocytes and a cell with a single Auer rod (cells with multiple Auer rods were not seen). MGG ×100. (By courtesy of Dr Dominic J. Culligan, Aberdeen.)

condensation and maturing granulocytes may include Pelger–Huët forms [124]. In one patient with t(5;17) the cytological features were more similar to M3 but Auer rods were lacking [124], whereas two previously reported cases had been associated with FAB M3 and M3 variant morphology, respectively. The t(11;17)(q13;q21); *NUMA1-RARA* rearrangement has been described as associated with hypergranular blasts with irregular nuclei, together with Pelger-like cells. One patient with AML associated with a *STAT5B-RARA* fusion gene was classified as FAB M1 AML but a minority of bone marrow blasts were considered suggestive of M3 variant AML [165]. A second patient was categorized as M3 AML [167]. A single patient with *PRKAR1A-RARA* fusion was morphologically M3 AML but without Auer rods [169].

The prognosis of M3-like AML associated with t(11;17)(q23;q21) is poor [47].

Immunophenotype The immunophenotype of AML with t(11;17)(q23;q21); *ZNF145-RARA* AML is similar to that of AML with *PML-RARA* [124]. There is usually expression of CD13 and CD33 and lack of expression of HLA-DR and CD34 [119,124]; CD56 is much more often expressed [124].

Cytogenetic and molecular genetic features All these molecular variants involve chromosome 17 and the *RARA* gene but the partner chromosome and gene differ. In t(11;17)(q23;q21) part of the *ZBTB16* gene (previously known as *ZNF145* (zinc finger 145) and *PLZF* (promyelocytic leukaemia zinc finger)) at 11q23 fuses with the *RARA* gene [162,164] to form a *ZBTB16-RARA* fusion gene on chromosome 11; there is a reciprocal *RARA-ZBTB16* fusion gene on chromosome 17. Both fusion genes are expressed and can be detected by RT-PCR [164]. Cases with a cryptic rearrangement (e.g. insertion of *RARA* into 11q23) leading to *ZBTB16-RARA* fusion are identical in other respects to those with t(11;17)(q23;q21) [124]. Other fusion partners of *RARA* are shown in Table 3.2; the rearrangement of *RARA* can be detected using dual-colour, break-apart FISH (see Fig. 3.16b).

Acute myeloid leukaemia with inv(16)(p13.1q22) or t(16;16)(p13.1;q22); CBFB-MYH11

Acute myeloid leukaemia associated with inv(16) was first described by Le Beau and colleagues in 1983 [171] although Arthur and Bloomfield had, shortly before, described AML with abnormal eosinophils in association with what they interpreted as del(16)(q22) [172]. Inversion of chromosome 16 (Fig. 3.18a) and the less common reciprocal

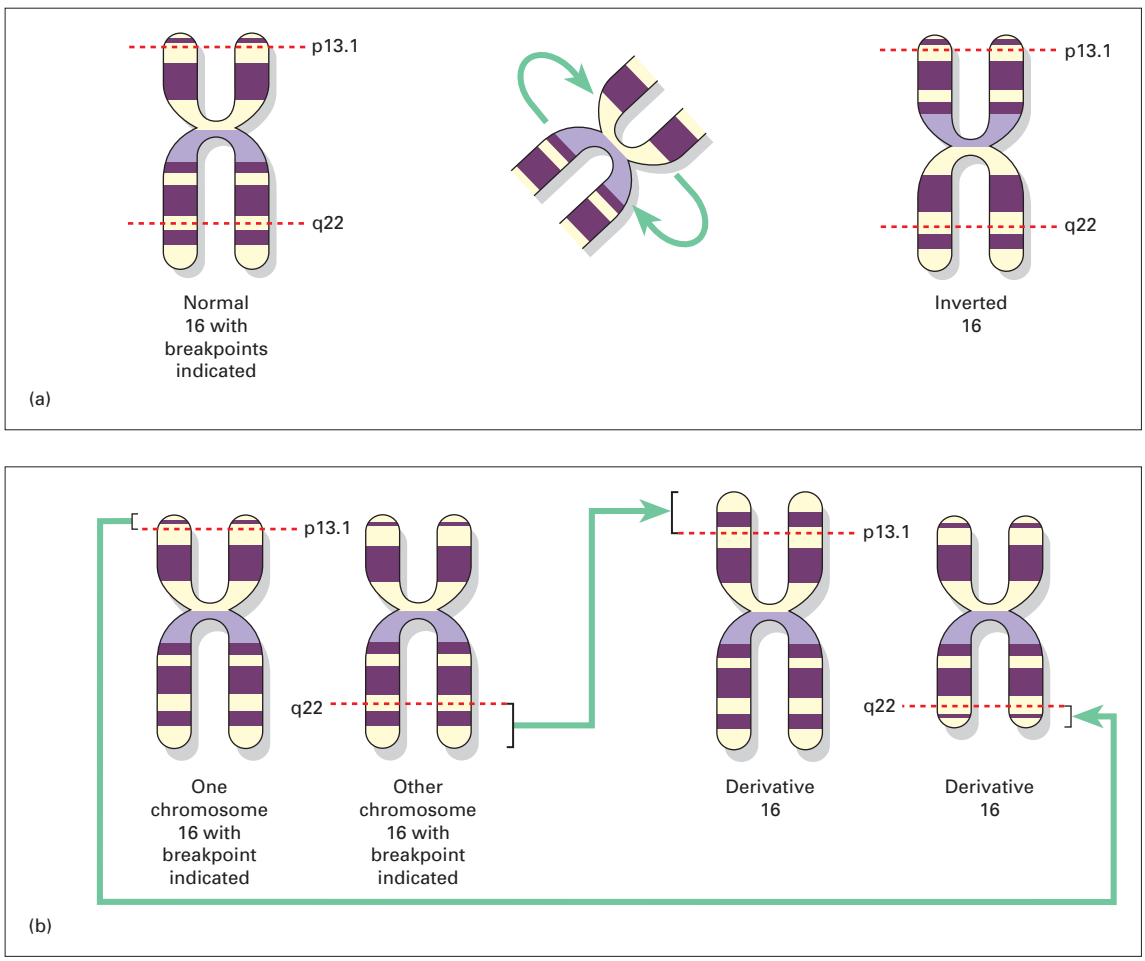


Fig. 3.18 (a) A diagrammatic representation of inv(16)(p13.1q22); this is an example of a pericentric inversion. (Modified from reference 2.) (b) A diagrammatic representation of t(16;16)(p13.1;q22).

translocation between the chromosome 16 pair (Fig. 3.18b) are associated with AML with identical features [173,174] although t(16;16) was associated with a worse prognosis in one study [175]. Such cases comprise 3–8% of AML with inv(16) being about five times as common as t(16;16); in a series of 1897 unselected adult patients prevalence was 4.6% [16]. The frequency is slightly higher in children than in adults [17] and in elderly adults the prevalence falls to 1% of cases of AML [18]. The prevalence is significantly higher among t-AML than among *de novo* AML [16] with therapy-related cases representing *c.* 14% of all cases [175]. Therapy-related cases are recognized following exposure to topoisomerase II-interactive drugs and other agents [22,23]. This subtype of leukaemia may have an antenatal origin with a latency of about 10 years [98]. A unique case has been reported in which lineage switch to B-lineage acute lymphoblastic leukaemia (ALL) occurred at relapse [176]. This inversion retains a good prognostic significance in patients in first relapse and is incorporated into the European Prognostic Index [48].

Clinical and haematological features This subtype of AML is associated with granulocytic and monocytic differentiation with cytologically abnormal eosinophils, which are often prominent, so that it is often referred to as 'M4Eo' AML [137,171,177] (Figs 3.19–3.23; see also Fig. 1.29). For convenience, the designation M4Eo will be used in the following paragraphs. However, it should be noted that a significant proportion of cases lack prominent eosinophilia, monocytic differentiation or both and are classified as FAB types M1, M2, M2Eo, M4 or M5. A small number of cases of M7 AML associated with inv(16) or t(16;16) have also been recognized [178]. Bone marrow eosinophils are less than 5% in more than a quarter of patients [179]. Nevertheless, morphologically abnormal cells of eosinophil lineage are probably always present, although they may constitute as little as 0.2% of bone marrow cells [47,179]. M4Eo AML is sometimes associated with meningeal leukaemia and with intracranial tumour formation by leukaemic cells. There have also been several reports of myeloid sarcoma of the bowel occurring in advance of overt leukaemia. Myeloid sarcoma may also be the first sign of relapse. There is a significant association with a tumour lysis syndrome [180]. Patients are relatively young (median age 34 years) [174].

Blast cells are variable in size and shape with prominent cytoplasmic basophilia. Some are monoblasts and some are primitive cells with occasional eosinophil granules. Bone marrow blasts cell are sometimes less than 20%; such cases are nevertheless categorized as AML [6]. Auer rods are usually present although only in a minority of cells; they are sometimes present in mature neutrophils. Neutrophils may be cytologically abnormal [179].

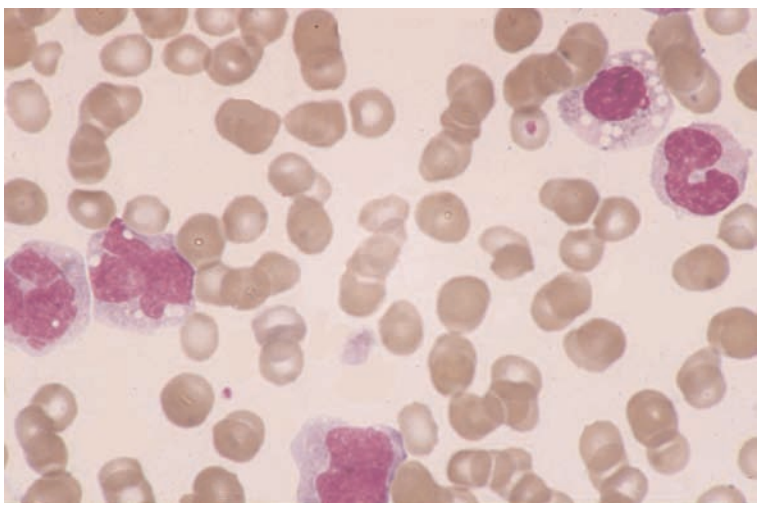


Fig. 3.19 PB film from a patient with FAB M4Eo AML associated with inv(16)(p13.1q22) showing four leukemic cells of monocyte lineage and an abnormal hypogranular vacuolated eosinophil with a non-lobulated nucleus. Often in this subtype of AML the PB eosinophils are normal. MGG ×100.

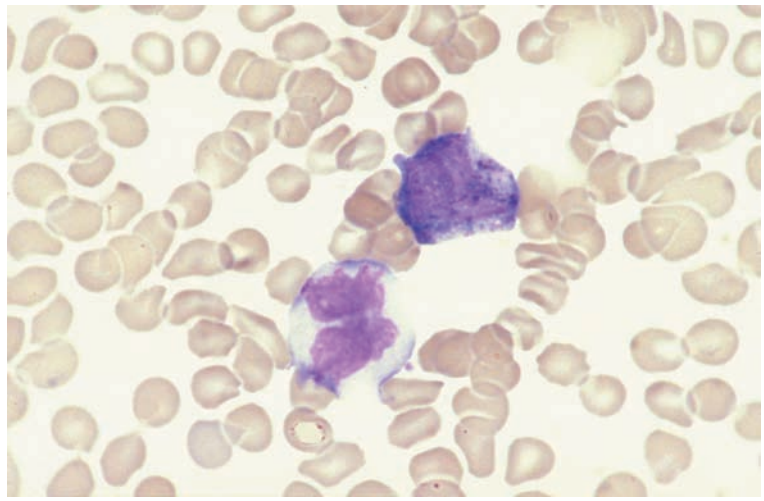
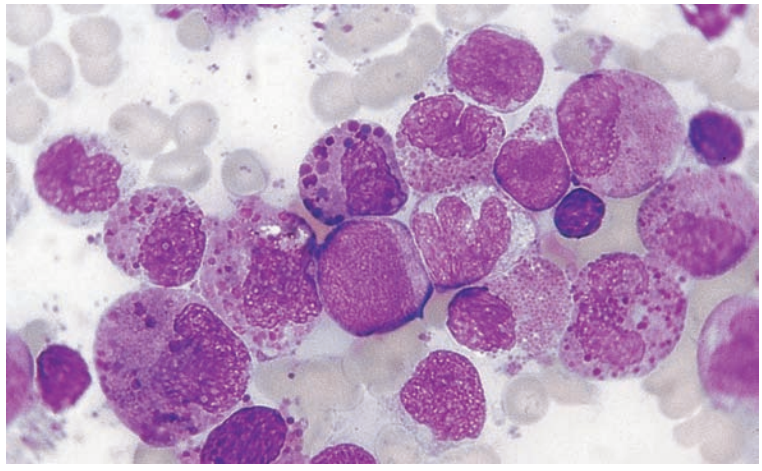
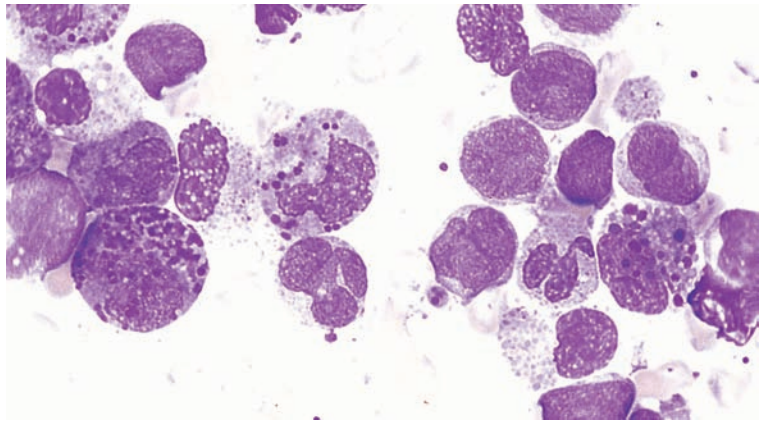


Fig. 3.20 PB film from a patient with FAB M4Eo AML associated with t(16;16)(p13.1;q22) showing an abnormal cell of monocyte lineage and an eosinophil precursor that has a mixture of granules with eosinophilic and basophilic staining characteristics. MGG ×100.



(a)



(b)

Fig. 3.21 BM film from a patient with AML FAB M4Eo/inv(16)(p13.1q22). (a, b) MGG stain showing abnormal blasts, monocytes, mature eosinophils and eosinophil myelocytes with abnormally basophilic granules. MGG ×100.

Continued

(c)

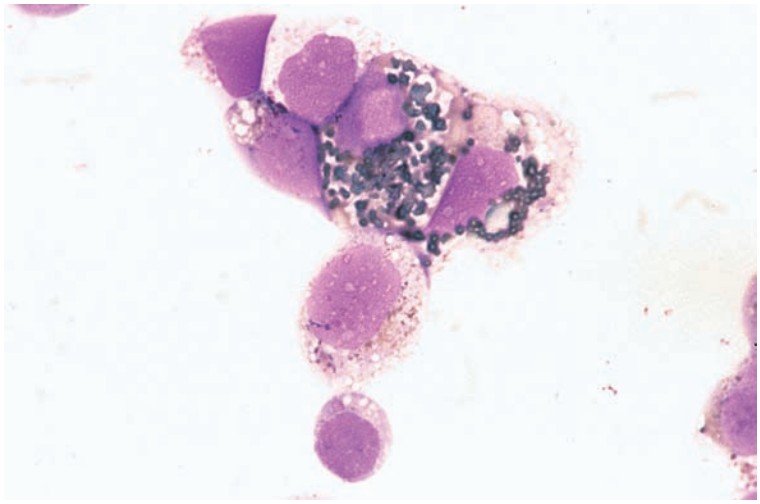


Fig. 3.21 (*Continued*) (c) SBB stain showing large abnormal granules in the eosinophil lineage and occasional small granules in monocytes. SBB ×100.

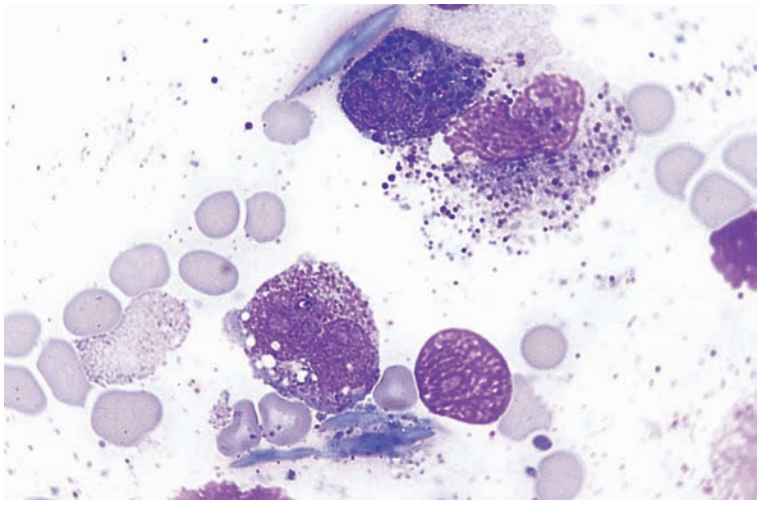


Fig. 3.22 BM film from a patient with AML M4Eo/inv(16)(p13.1q22) showing Charcot–Leyden crystals and abnormal cells of eosinophil lineage. MGG ×100. (By courtesy of Dr Ralph Cobcroft and Dr Devinder Gill, Brisbane.)

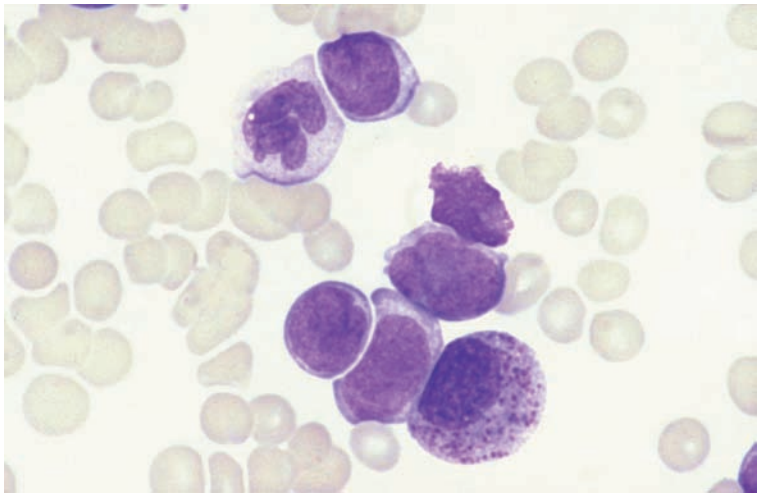


Fig. 3.23 BM film from a patient with FAB M4Eo AML and t(16;16)(p13.1;q22) showing one abnormal monocyte, four blast cells and an eosinophil precursor with granules with mixed eosinophilic/basophilic staining characteristics. MGG ×100.

Bone marrow eosinophils and to a greater extent eosinophil myelocytes show prominent pro-eosinophilic granules, which are basophilic in their staining characteristics (see Fig. 3.19). Mature eosinophils may be hypolobulated. In some cases the eosinophils have unusually large and folded nuclei. Eosinophils also show aberrant cytochemical reactions. Some have PAS-positive granules and some give positive reactions for CAE. PAS-positive granules are not specific for this subtype of leukaemia nor in fact for leukaemic eosinophils since they may be observed in t(8;21)/M2 [11] and sometimes in reactive eosinophilia [137]. Positivity for CAE may, however, indicate that eosinophils are part of a leukaemic process [137]. Despite the bone marrow eosinophilia, peripheral blood eosinophilia is unusual and blood eosinophils are usually morphologically normal. Cytogenetic analysis has confirmed that eosinophils are indeed part of the abnormal clone. Occasional patients with M4Eo/inv(16) have had increased bone marrow basophils and basophil precursors (confirmed by metachromasia with toluidine blue) [181] (see Fig. 1.29). Despite the monocytic differentiation, non-specific esterase reactions are often weak [179]. Dysplasia of cells of erythroid and megakaryocyte lineages has been regarded as unusual but in a study of 21 patients was observed to be usually present and to be unrelated to either the presence of additional cytogenetic abnormalities or to poor prognosis [182].

The prognosis of this category of AML is relatively good with reported complete remission rates of 85–93% [28,174] and 5-year survivals of 50–60% [18,26–28,174]. A series of 266 patients entered into MRC trials had a median 10-year survival of 55% [29]. Prognosis is significantly better in those with a presenting WBC of 20×10^9/l or less [31] and is worse in those with a WBC above 120×10^9/l or a platelet count below 30×10^9/l [174]. The prognosis in children appears to be equally good [47]. In view of the relatively good prognosis, stem cell transplantation in first remission is generally considered to be not indicated; in one large group of patients the survival was no better with transplantation than with standard chemotherapy [174]. Intensive post-remission intensification with high dose cytarabine was thought to contribute to improved survival in one trial [46] but not in another [174]. Five-year survival in therapy-related cases approaches 50% and is thus considerably better than in most other types of t-AML [100].

Immunophenotype Flow cytometry immunophenotyping shows both granulocytic and monocytic differentiation [49] (Fig. 3.24). The plot of sideways light scatter against forward light scatter may show a characteristic forked pattern [122]. Typically there is positivity for CD13, CD33, CD14, CD15, CD64, CD65, CD117, MPO (strongly expressed) and HLA-DR while CD11b is positive in about a third of cases [183]. Antigen expression may show considerable heterogeneity with a population of blasts expressing CD34 but little CD11b, CD14, CD15 or CD64, a population of maturing monocytes expressing CD4, CD33, CD11b, CD11c, CD14, CD64 and lysozyme, and a population of granulocytes with considerable sideways light scatter and expression of CD13, CD33, CD65, MPO and strong CD15 [122]. CD2 is expressed in about 40% of patients and correlates with CD11b and CD14 expression [54,121,130]. CD19 may be expressed. CD34 is expressed in the great majority of cases [184,185]. TdT is more often positive than in AML in general [186]. CD7 is not usually expressed [185]. Strong expression of CD34 and CD13 and weak expression of CD33 has been reported to have a reasonably high sensitivity and specificity [186]. This category of leukaemia has also been identified by flow cytometry, using an antibody directed at the CBFB-MYH11 fusion protein [187].

Cytogenetic and molecular genetic features M4Eo AML is associated both with inv(16)(p13.1q22) (Fig. 3.25) and, less often, t(16;16)(p13.1;q22) (Fig. 3.26). The molecular mechanism of leukaemogenesis is the same. The commonest secondary chromosomal abnormalities are trisomy 22, trisomy 8, trisomy 21, trisomy 9 and del(7q); trisomy 22 and del(7q) are uncommon in association with other specific chromosomal aberrations. The presence of secondary cytogenetic abnormalities, even complex ones, did not appear to worsen prognosis in one study [27] but, in another, trisomy 21, present in about 4% of patients, was associated with shortened survival [175], in a third, trisomy 22 was a favourable feature [188] and in yet another study of patients with either t(8;21) or inv(16), complex abnormalities, present in 12% of patients with inv(16), were

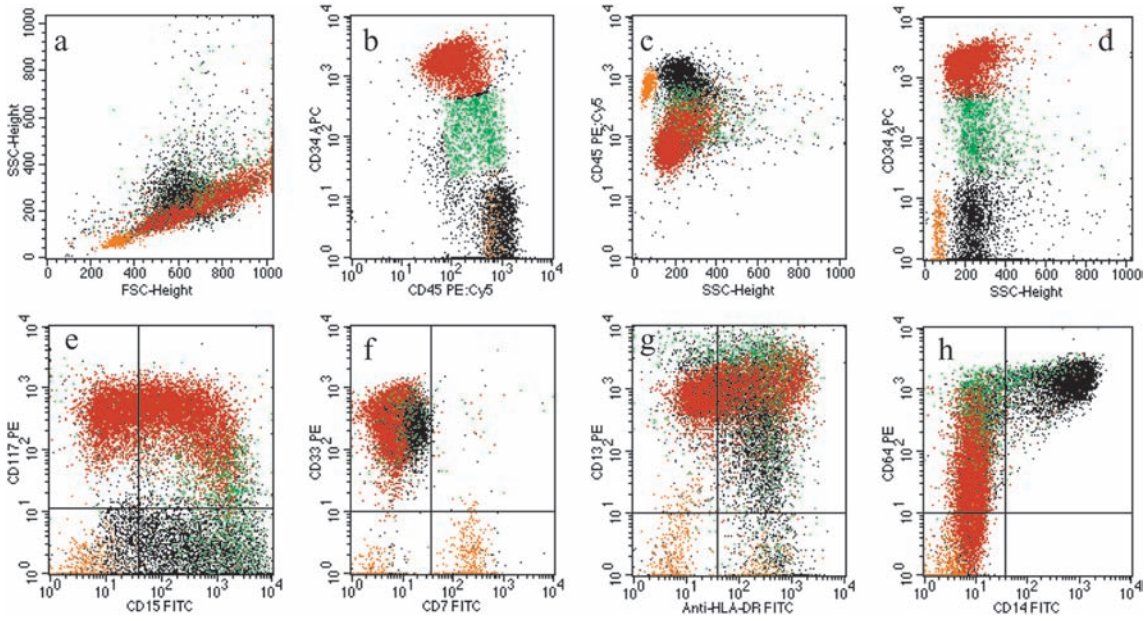


Fig. 3.24 Four-colour flow cytometry immunophenotyping in a patient with AML associated with inv(16) (compare with Fig. 2.3). Plots a–d show distinct CD34++, CD34+ and CD34– blast cell populations. Plots e and h show a spectrum of differentiation, with the most immature blast cell component (plot e) being CD34++ CD117+ CD15+/–. The mature monocytic component (CD34–) is CD14+ CD64+ CD15+ (plot h) with high levels of CD45 expression, comparable to that of normal lymphocytes. Blast cells show a continuous spectrum of maturation but at all stages of maturation there is evidence of monocytic differentiation (ranging from CD64+ to CD64+++ CD14+). The blast cells continue to be CD33 and CD13 positive at all stages of differentiation (f and g). (By courtesy of Dr Steve Richards.)

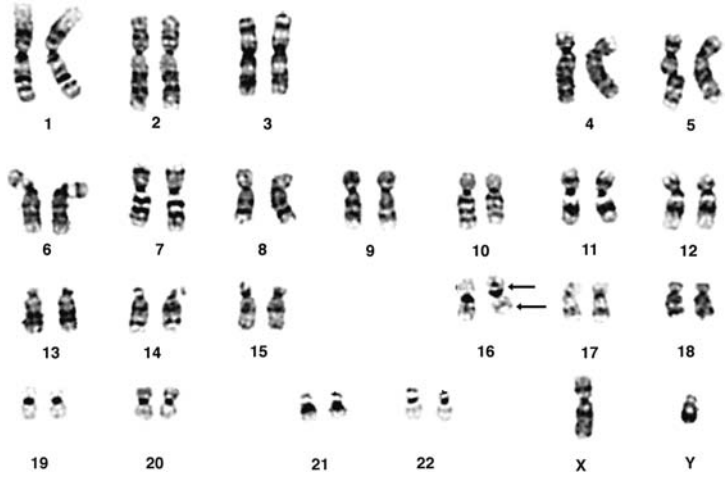


Fig. 3.25 A karyogram showing inv(16)(p13.1q22). (By courtesy of Dr Fiona Ross, Salisbury.)

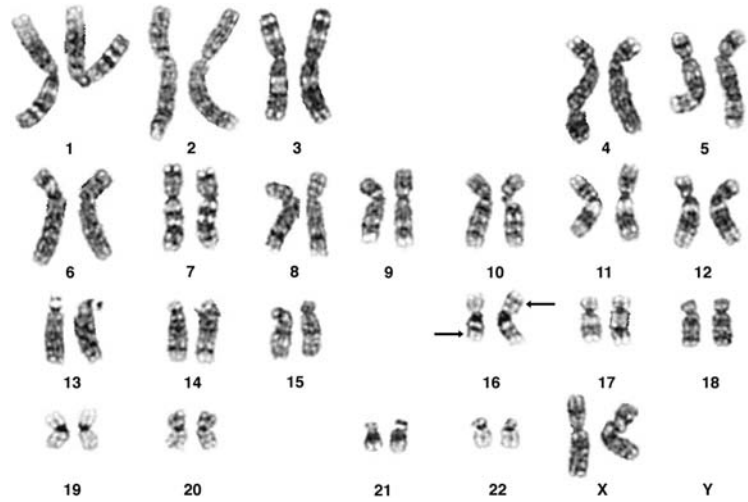


Fig. 3.26 A karyogram showing t(16;16)(p13.1;q22). (By courtesy of Dr Fiona Ross.)

associated with a worse prognosis [28]. A definitive answer is provided by the outcome of 266 patients entered into MRC trials; a lower white cell count and a significantly longer survival was found in patients with additional abnormalities, including specifically those with trisomy 22 [29]. The detection of trisomy 22 in a patient who appears to lack inv(16) is an indication for molecular analysis for the *CBFB-MYH11* fusion gene since a cryptic chromosomal rearrangement may be present [189]. M4Eo AML has also been described in association with a deletion of chromosome 16, del(16)(q22) [172]. Most such cases have been considered to show the same features as are seen with inv(16) but there have been reports suggesting that patients with del(16)(q22) have M4 AML without eosinophilia [137,190] and with a high incidence of preceding MDS [190]. Both inv(16) and del(16) are relatively difficult to detect by conventional cytogenetic analysis, particularly if metaphases are not of high quality, and it can also be difficult to distinguish between the various abnormalities of chromosome 16 [137]. It may be that some of the cases reported as del(16) are actually examples of inv(16) while others, without the typical features of M4Eo AML, may have a different mechanism of leukaemogenesis. Because of this uncertainty, molecular analysis is indicated in patients who appear to have del(16). M4Eo AML has also been associated with a variant translocation, t(5;16)(q33;q22).

The molecular mechanism of leukaemogenesis is fusion of part of the *CBFB* (core binding factor β) gene at 16q22 with part of the *MYH11* (myosin heavy chain 11) gene at 16p13 to form a fusion gene, *CBFB-MYH11*, which encodes a protein that interferes with normal control of transcription [191]. The reciprocal fusion gene, *MYH11-CBFB*, is expressed in only a proportion of cases. The precise breakpoints differ at a molecular level between *de novo* and therapy-related cases [149]. A minority of patients have submicroscopic deletion of part of chromosome 16, telomeric to the *CBFB* gene [192]; this may be associated with a worse prognosis. A *KIT* mutation is found as a secondary abnormality in a significant proportion of patients with inv(16), some of whom have aberrant bone marrow mast cells [80]; such mutations correlated with a worse prognosis in one series of patients [78] but not in another [77]. *KIT* and *RAS* mutations are both more common than in AML in general [81–83]. *NRAS* mutation, present in 37% of patients, did not influence prognosis [83]. *KIT* mutations, present in about 30% of patients, have been associated with a worse prognosis [6]. *FLT3*-ITD is uncommon [73,78,157]. In one large study *FLT3*-ITD was found in 7% of patients and *FLT3*-TKD mutation in 24% [75].

Using microarray analysis, cases of AML with inv(16)/t(16;16) can be distinguished from cases with t(8;21) or t(15;17) [72].

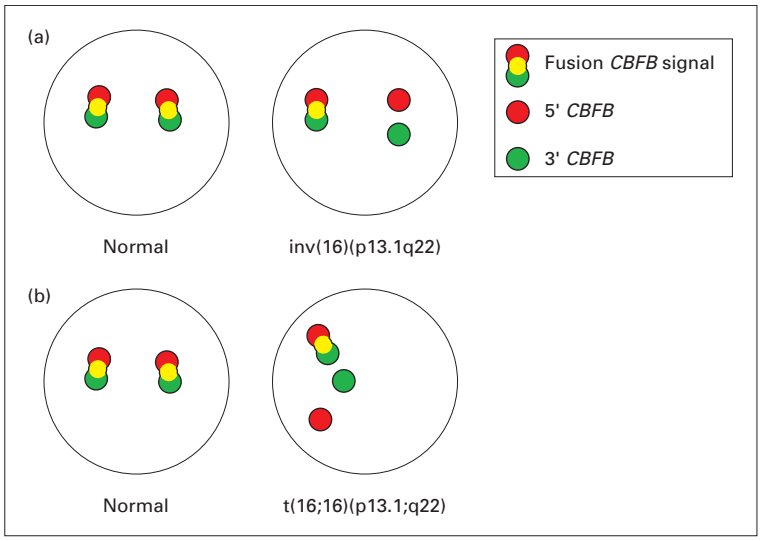


Fig. 3.27 Diagrammatic representation of a dual-colour, break-apart FISH technique for detecting the disruption of *CBFB* in inv(16)(p13.1q22) and t(16;16)(p13.1;q22), using a red-labelled probe for 5′ *CBFB* and a green-labelled probe for 3′ *CBFB*: (a) a normal cell has two yellow fusion signals whereas a cell with inv(16) has one normal fusion signal and separated red and green 5′ and 3′ *CBFB* signals on the two arms of the inverted chromosome; (b) a cell with a t(16;16) has one normal fusion signal on the q arm and a distinct green 3′ *CBFB* signal on the p arm of the same chromosome; the other chromosome 16 has a discrete red 5′ *CBFB* signal.

Both inv(16)(p13.1q22) and t(16;16)(p13.1;q22) can be detected by single-colour FISH using a chromosome 16 short arm paint [193], by dual-colour, break-apart FISH using a 5′ *CBFB*–3′ *CBFB* probe (Figs 3.27 and 3.28) and by dual-colour, dual-fusion FISH using probes for *CBFB* and *MYH11*. FISH is useful for the detection of masked inv(16) in patients with simple variant translocations involving chromosome 16 and diverse partners. Both inv(16) and t(16;16) can also be detected by RT-PCR for *CBFB-MYH11*; two primer sets are needed because of the variability of breakpoints [194]. A significant proportion of patients (varying from less than 10% to 50% in different series of patients [195]) have been reported to have no detectable abnormality of chromosome 16 despite having the same molecular genetic abnormality demonstrable by RT-PCR. However, in one large study all 27 cases were identified by conventional cytogenetic analysis [15] and another group also found no discrepancy [196]. When molecular techniques are employed the prevalence of this subtype among cases of AML may be as high as 10% [177]. Cases that do not have the typical M4Eo cytological features are more likely to be missed on conventional cytogenetic analysis.

Detection of MRD does not necessarily presage haematological relapse but detection above a certain level by RQ-PCR has been found predictive

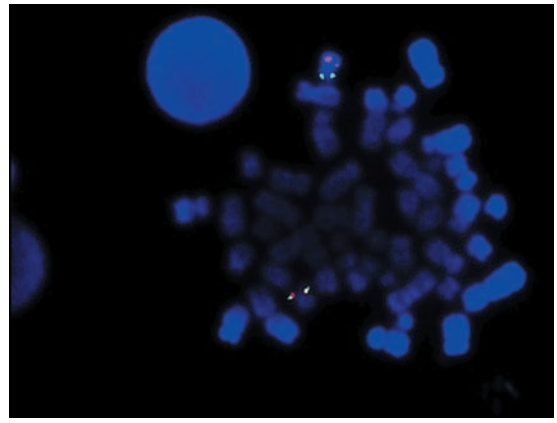


Fig. 3.28 FISH of a metaphase from a patient with inv(16)(p13.1q22) using a dual-colour, break-apart probe; the 5′ *CBFB* probe (centromeric to the breakpoint) is labelled red and the 3′ *CBFB* probe (telomeric to the breakpoint) is labelled green. Normal cells will thus have two fused red–green (yellow) signals. The leukaemic cell shown has a chromosome 16 with an inversion (top) showing separate red and green signals (the double signals represent the two chromatids). The normal chromosome 16 (bottom) has two normal fusion signals. (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

[197,198]. Using RQ-PCR, the quantity of fusion gene transcript present at presentation and after consolidation chemotherapy is predictive for event-free survival [87]. A 1 log or greater increase in transcript from the remission level is predictive of relapse [88].

Acute myeloid leukaemia with t(9;11)(p22;q23); MLLT3-MLL

There is a strong association between acute monoblastic/monocytic leukaemia and deletions or translocations with a breakpoint within the *MLL* gene (a homologue of the *Drosophila trithorax* gene previously known as *HRX*, *ALL-1* and *Htrx-1*) at 11q23 [199]. Overall such cases comprise about 3–4% of adult AML cases [14] but about 18% of childhood cases [17]; in a series of 1897 unselected adult patients prevalence was 2.8%, being significantly higher among t-AML cases [16]. In another series of patients about 8% of those with t(9;11) had AML secondary to topoisomerase II-interactive drugs [200]. Of the myeloid leukaemias in which there is a chromosomal rearrangement with an 11q23 breakpoint, that associated with t(9;11)(p22;q23) is the most common.

Clinical and haematological features The prevalence of AML with t(9;11)(p22;q23) is highest among children and infants (including babies with congenital leukaemia) but adult cases also occur. In one large series of children with AML, 7% were found to have t(9;11) [17]. Soft tissue tumours of blast cells may be particularly common, t(9;11) being the most frequently observed karyotypic abnormality in one series of such patients [201]. Most cases of AML with t(9;11) are FAB M5a AML (Fig. 3.29) but some are M5b or M4 (Fig. 3.30) and occasional cases are M1, M2 or M7 [200]. Cytological and cytochemical features are those expected for these FAB categories. Auer rods are quite uncommon. Two patients with either t(9;11) or *MLLT3-MLL* have been considered to have acute leukaemia of dendritic cell origin [202]. Cases of ALL also occur.

The prognosis has varied considerably between different series of patients. Two studies found t(9;11) to be a good prognostic factor in infants and children, respectively [203,204]. Disease outcome in adults has been less consistent. In adults, AML associated with t(9;11) is favourable with regard to the probability of complete remission but intermediate or poor with regard to overall survival [27,16]. Remission rate is 80–85% but with a 5-year survival of around 20–40% [27,47,205]. In one series of patients, median survival was only 10 months, and it was suggested that this category of AML should be regarded as poor prognosis rather than intermediate [16]. However, a series of 56 adult patients entered into MRC trials fell into the intermediate prognostic group with a 10-year survival of 39%; other patients with t(6;11) and t(10;11), also with an 11q23 breakpoint, had a worse outcome [29]. A French

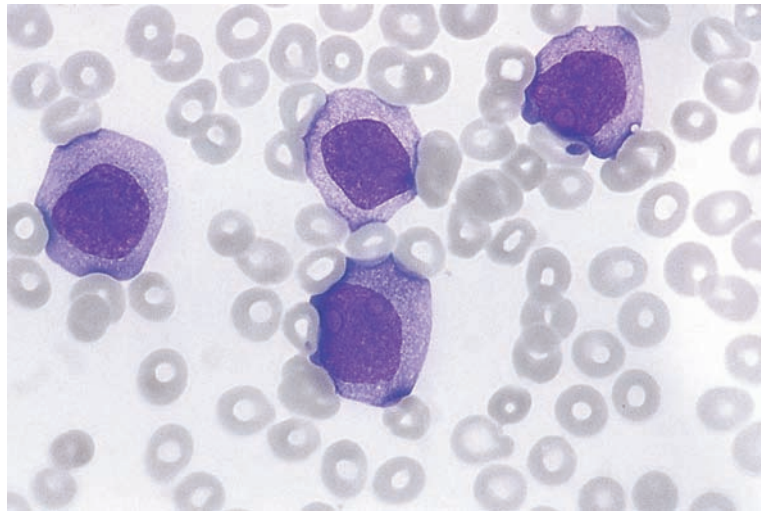


Fig. 3.29 PB film from a patient with FAB M5a AML associated with t(9;11)(p22;q23). MGG ×100.

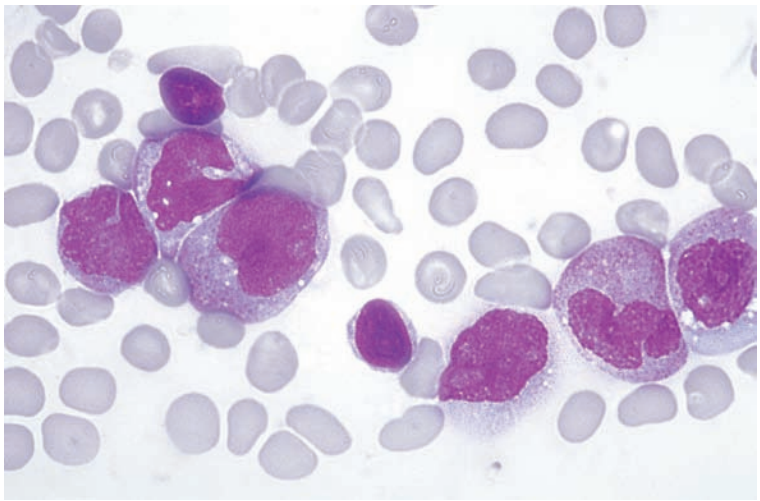


Fig. 3.30 BM film from a patient with FAB M4 AML associated with t(9;11)(p22;q23). MGG ×100. (By courtesy of the European 11q23 Workshop [201].)

study confirmed the poor prognosis of t(6;11) and found a better prognosis for children with t(9;11) in comparison with adults [205]. The prognosis of therapy-related cases (classified as t-AML in the WHO classification) is worse than that of *de novo* cases, with less than 20% 5-year survival [16,206]. Different outcomes in different series of patients could be the result of the use of different chemotherapeutic agents since cytarabine was a major component of the treatment in one study with a particularly good outcome [81]. There is *in vitro* evidence that this subtype of leukaemia may be particularly sensitive not only to cytarabine but also to etoposide and anthracyclines and in addition may show sensitivity to vincristine and asparaginase [81].

Immunophenotype The immunophenotype is characteristic of M5 AML. CD4, CD15, CD33, CD64, CD65 and HLA-DR are usually expressed. CD13 was found to be usually negative in three series of patients [51,196] but positive in two thirds of patients in another [200]. Similarly, CD14 was usually negative in three series of patients [51,196] but was positive in half the cases in a fourth series [200]. CD11b and 11c are often expressed [200] and there may also be expression of CD64 and lysozyme [6]. CD34 and CD7 were positive in 40–50% of cases in one study [200] whereas three further series found CD34 expression in less than 30% of patients [196]. NG2, a chondroitin sulphate proteoglycan, is expressed. This immunophenotypic marker was initially reported to be expressed in AML associated with t(9;11) or other *MLL* rearrangement but not in AML without *MLL* rearrangement [207]. However, subsequently expression, together with CD56 expression, was demonstrated in cases with monocytic differentiation but without *MLL* rearrangement [208].

Cytogenetic and molecular genetic features t(9;11)(p22;q23) is difficult to detect by conventional cytogenetic analysis since the alteration in the banding pattern is subtle (Figs 3.31 and 3.32). Detection is facilitated by dual-colour, break-apart FISH with a *MLL* probe. The commonest secondary abnormalities associated with t(9;11) are trisomy 6, trisomy 8, trisomy 8q, trisomy 19 and duplication of the derivative chromosome 9 [200,209,210]. *MLL* rearrangements can be detected by Southern blotting and *MLLT3-MLL* fusion by RT-PCR. t(9;11) can also be detected by two-colour FISH using probes for *MLL* and *MLLT3*. *FLT3* mutations are less common than in AML in general [73].

Acute myeloid leukaemia with other MLL rearrangement

In the 2008 WHO classification, other subtypes of AML with *MLL* rearrangement are regarded as distinct from AML with *MLLT3-MLL*. Some are categorized as t-AML, some as AML with myelodysplasia-related changes and the remainder as AML,

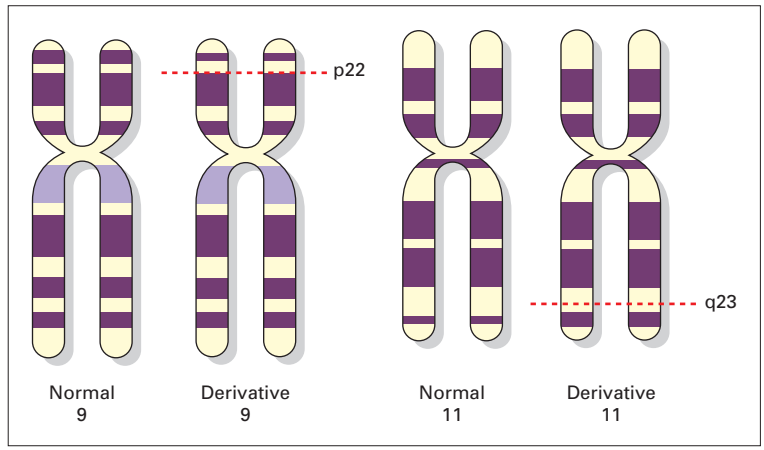


Fig. 3.31 Diagrammatic representation of t(9;11)(p22;q23); the 9p breakpoint is reported variably as p21 or p22. (Modified from reference 2.)

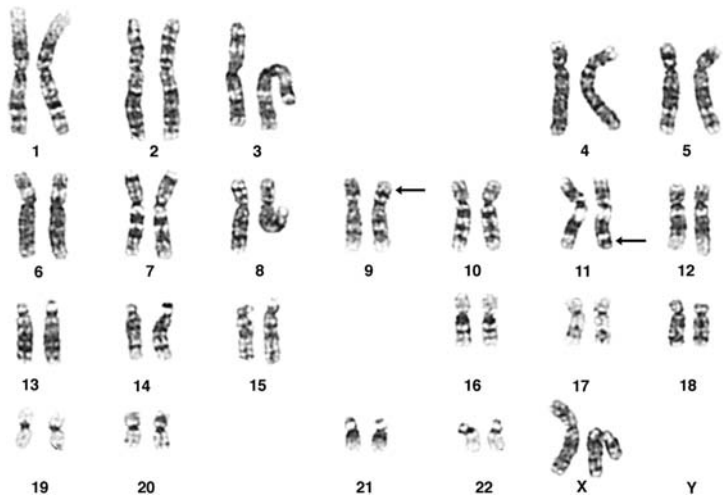


Fig. 3.32 A karyogram showing t(9;11)(p22;q23). (By courtesy of Dr Fiona Ross.)

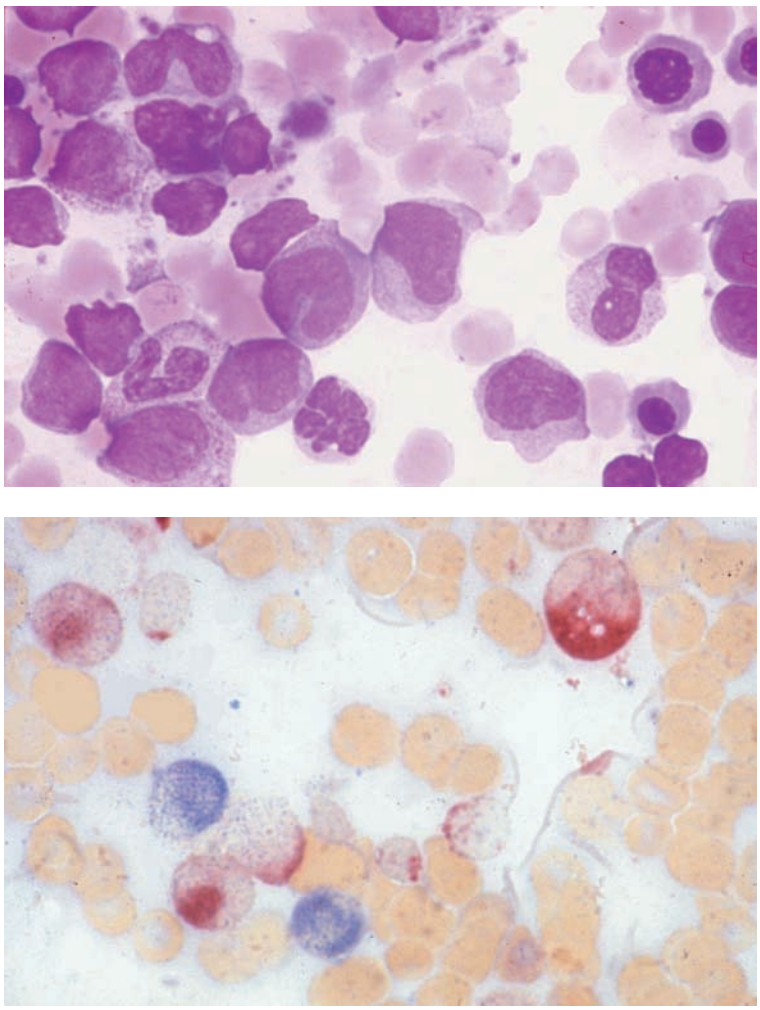
not otherwise specified. The more common of these are shown in Table 3.3 [211–215] and other less common subtypes in Table 3.4 [47,203,211,213, 214,216–230]. One reported case of AML with t(10;11) was considered to be of dendritic cell origin [202]. These subtypes of AML with an 11q23 breakpoint have some characteristics in common with each other and with AML associated with t(9;11) but they differ in other important features. Molecular mechanisms of leukaemogenesis differ.

Clinical and haematological features Common characteristics include a predominance of FAB M4 and M5 AML and a possible relationship to topoisomerase II-interactive drugs. Congenital cases have been reported in association with t(4;11)(q21;q23) (Fig. 3.33), t(10;11)(p13;q23) and t(11;19)(q23;p13.1) (Fig. 3.34) [231]. Features that differ between categories include the relative proportions of AML, ALL and mixed phenotype acute leukaemia, the relative proportions of *de novo* and secondary cases, the proportion of patients presenting with MDS and the relative frequency of different FAB subtypes of AML [211–215]. Prognosis also differs but is generally poor [27,232]. The prognosis in cases with t(10;11)(p12;q23) is variously reported as poor or intermediate [29,233]. The prognosis of AML associated with t(6;11)(q27;q23) is poor [29,234].

Table 3.3 Characteristics of acute leukaemia and related conditions associated with translocations and deletions involving 11q23 studied at the European 11q23 Workshop (percentages are shown in parentheses).

Translocation or other rearrangement	Total	Infants (less than 12 months)	Children (1–14 years)	ALL	AML	Main FAB category of AML	Other acute leukaemia*	MDS	t-AML/ t-MDS	Reference
t(4;11)(q21;q23)	183	63 (34)	36 (20)	173 (95)	6 (3)	M4	4 (2.2)	Nil	10 (5.5)	[211]
t(6;11)(q27;q23)	30	2 (7)	6 (20)	3 (10)	27 (90)	M4/M5a	Nil	Nil	Nil	[212]
t(9;11)(p21–22;q23)	125	21/123 (17)	46/123 (37)	9 (7)	108 (86)	M5a	3 (2)	5 (4)	10 (8)	[200]
t(10;11)(p12–22;q23)	20	6/19 (32)	10/19 (53)	4 (20)	15 (75)	M5a	1 (5)	Nil	1 (5)	[213]
t(11;19)(q23;p13.1)	21	3 (14)	1 (5)	Nil	19 (90)	M4	Nil	2 (10)	7 (33)	[214]
t(11;19)(q23;p13.3)	32	13 (41)	7 (22)	21 (66)	7 (22)	M4 or M5a	4 (12)	Nil	Nil	[214]
del(11)(q23)	57	3 (5)	10 (33)	27 (47)	16 (28)	M4, M5a, M5b	2 (4)	12 (21)	1 (2)	[215]

ALL, acute lymphoblastic anaemia; AML, acute myeloid leukaemia; FAB, French–American–British (classification); MDS, myelodysplastic syndrome; t-AML, therapy-related AML; t-MDS, therapy-related MDS.
* Other acute leukaemia = acute biphenotypic, acute stem cell and acute unclassified leukaemia.



(a)

(b)

Fig. 3.33 BM film from a patient with FAB M4 AML associated with t(4;11)(q21;q23) showing positivity for both chloroacetate esterase (blue reaction product) and α-naphthyl acetate esterase (brown reaction product). This translocation is more often associated with pro-B ALL. (a) MGG ×100. (b) Mixed esterase reaction, ×100.

Table 3.4 Some of the less common subtypes of acute myeloid leukaemia* with an 11q23 breakpoint involving the *MLL* gene† [47,203,211,213,214,216–230].

Translocation or other rearrangement	Type of leukaemia	Molecular event	Reference
t(1;11)(p32;q23)	FAB M5	*MLL-EPS15* (*AF1p*)	
t(1;11)(p36;q23)	FAB M5 (congenital)		[216]
t(1;11)(q21;q23)	FAB M4 (infants)	*MLL-MLLT11* (*AF1q*)	
t(2;11)(p21;q23)		Not known	
t(3;11)(p21;q23)	t-AML (FAB M5)	*MLL-AF3p21*	
t(3;11)(q25;q23)	t-AML (FAB M4)	*MLL-GMPS*	[217]
t(3;11)(q28;q23)	A case of FAB M5 AML (t-AML)	*LPP-MLL* and *MLL-LPP*	[218]
t(4;11)(p12;q23)	t-AML	*MLL-FRYL* (*AF4p12*)	[219]
t(4;11)(q21;q23)	FAB M4 (more often ALL)	*MLL-MLLT2*	[211]
t(4;11)(q31;q23)		Not known	
t(6;11)(q21;q23)		*MLL-FOXO3A* (*AF6q21*)	
t(6;11)(q27;q23)	FAB M4 or M5	*MLL-MLLT4* (*AF6*)	
t(8;11)(q24;q23)	FAB M5	Not known	[220]
t(9;11)(q21;q23)‡		Not known	
t(9;11)(q22;q23)‡		Not known	
t(10;11)(p12;q23)	FAB M5a	*MLL-MLLT10* (*AF10*)	[213]
t(10;11)(p13-14;q23)	FAB M0, M1	*MLL-PICALM*	[221]
t(10;11)(p11.2;q23)	AML	*MLL*-SSH3BP1 (*ABI1*)	
t(10;11)(q22;q23)	FAB M4		[222]
ins(10;11)(p11;q23q13-24)		Not known	
ins(11;9)(q23;q34)inv(11)(q13q23)	A case of FAB M4 AML	*MLL-FNBP1* (*FBP17*)	[222]
t(11;11)(q13;q23)		Not known	
inv(11)(p15q23)		Not known	
inv(11)(q14.2q23.1)	A case of AML	*MLL-PICALM* (*CALM*)	[223]
t(11;12)(q23;p13)		Not known	
t(11;14)(q23;q24)	A case of FAB M5 AML	*MLL-GPHN* (*gephyrin*)	[224]
t(11;15)(q23;q12)		Not known	
t(11;15)(q23;q14)	A case of AML	*MLL-AF15q14*	[225]
t(11;16)(q23;p13)		*MLL-CREBBP* (*CBP*)	
cryptic t(11;17)(q23;p13)	FAB M4 (t-AML)	*MLL-GAS7*	[226]
t(11;17)(q23;q12-21)	FAB M5 AML	*MLL-MLLT6* (*AF17*)	
t(11;17)(q23;q25)	t-AML	*MLL-SEPT9* (AF17q)	
t(11;19)(q23;p13.1)	FAB M5	*MLL-ELL*	[214]
t(11;19)(q23;p13.3)	FAB M5 (also B-lineage and T-ALL)	*MLL-MLLT1* (*ENL*)	[214]
t(11;19)(q23;p13)	AML	*MLL-SH3GL1* (*EEN*)	[227]
t(11;19)(q23;q12 or q13)		Not known	
t(11;21)(q23;q13)		Not known	
t(11;22)(q23;q11)		*HCDCREL-MLL*	
t(11;22)(q23;q13)	AML	*MLL-EP300* (*P300*)	[228]
t(X;11)(q13;q23)	FAB M4 or M5	*MLL-MLLT7* (*AFX1*)	
t(X;11)(q24;q23) – often complex	FAB M2	*MLL-SEPT6* (*KIAA01*)	[229]
Tandem partial duplication of *MLL*, with or without trisomy 11	FAB M0, M1, M2, M4, M7	*MLL*-PTD	[230]

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; FAB, French–American–British (classification); PTD, partial tandem duplication; t-AML, therapy-related AML.

* Some of these translocations are also associated with ALL or biphenotypic acute leukaemia suggesting that the mutation occurs in a pluripotent stem cell; in the case of t(4;11) the majority of cases are of ALL.

† Rearrangement of 11q23 may be detected by conventional cytogenetic analysis. Rearrangement of the *MLL* gene can be detected in a larger number of cases by fluorescence *in situ* hybridization (FISH), the reverse transcriptase polymerase chain reaction (RT-PCR) or Southern blotting. FISH with a dual-colour, break-apart MLL probe is particularly useful, the fused signal being split when the gene is rearranged; this method is independent of prediction of the partner chromosome. Deletion of chromosome 11, interpreted either as terminal or interstitial, has also been associated with M4 or M5 AML (and with MDS), but FISH analysis suggests that many apparent deletions are actually reciprocal translocations.

‡ To be distinguished from the more frequent t(9;11)(p21;q23), *MLLT3-MLL*.

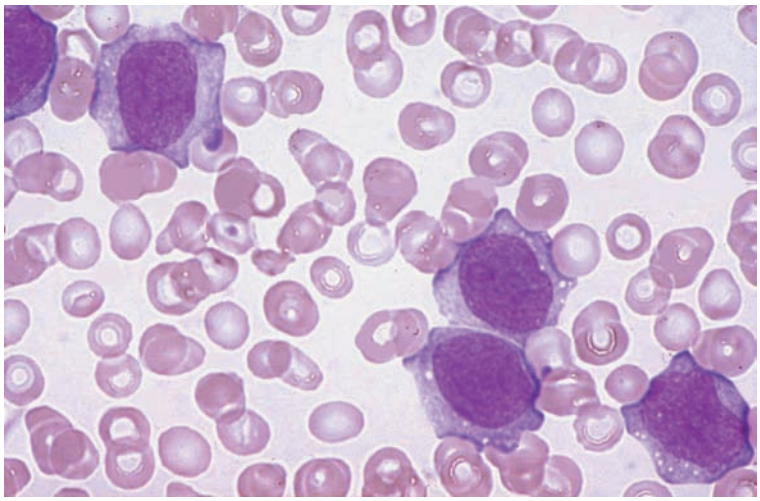


Fig. 3.34 BM film from a patient with acute monoblastic leukaemia (FAB M5a AML) associated with t(11;19)(q23;p13.1) showing monoblasts. MGG ×100.

Immunophenotype The immunophenotype is generally similar to that of AML associated with t(9;11). NG2 expression has been demonstrated in cases with *MLL* rearrangement associated with t(11;17)(q23;q21), t(11;19)(q23;p13), other chromosomal abnormality without cytogenetic evidence of an 11q23 abnormality, and a normal karyotype [207].

Cytogenetic and molecular genetic features Cytogenetic analysis underestimates the frequency of cases with 11q23 rearrangement in comparison with molecular analysis [232]. FISH analysis, e.g. using dual-colour, break-apart FISH or multicolour FISH, permits detection of a higher proportion of cases but some cases are detected only by Southern blot or other deoxyribonucleic acid (DNA) analysis [232]. The *MLL-MLLT4* (previously *MLL-AF6*), *MLL-MLLT10* (previously *MLL-AF10*) and *MLL-MLLT1* (previously *MLL-ENL*) fusion genes can be detected by RT-PCR, providing not only confirmation of diagnosis but also a possible target for detection of MRD [197]. In the case of *MLL-MLLT1*, RT-PCR detects more cases than are detected by conventional cytogenetic analysis [235]. Multiplex PCR can be used to detect the more common *MLL* rearrangements in a single reaction. RQ-PCR can be used to detect *MLL* duplication.

Acute myeloid leukaemia with t(6;9)(p23;q34); DEK-NUP214

Cases of leukaemia associated with t(6;9)(p23;q34) (Fig. 3.35) comprise less than 1% of cases of AML.

Clinical and haematological features AML associated with t(6;9)(p23;q34.3) may be therapy-related or develop *de novo*. Therapy-related cases, categorized as t-AML in the WHO classification, can follow exposure to either topoisomerase II-interactive drugs [22] or alkylating agents. Patients tend to be young but, despite this, the prognosis is poor; the complete remission rate is around 40% with the 5-year survival being very low [27,236,237]. A series of 34 patients entered into MRC trials had a 10-year survival of 26% [29].

Cases are usually FAB M2 AML, less often M4 and least often M1 [236–238]. Trilineage myelodysplasia is common [239,240] and overt AML may be preceded by MDS. The presenting WBC is generally low and there may be pancytopenia [6]. Blasts are usually granular [240]. The blast cell percentage may be relatively low (e.g. between 20% and 30%) [240]. Auer rods are reported as sometimes [240] or often [236,237] present. Bone marrow basophilia is common (Fig. 3.36) but not invariable and may also be present during a preceding myelodysplastic phase [236]. The peripheral blood basophil count is also often elevated [236]. Basophilic differentiation can be confirmed by metachromatic staining with toluidine blue (Fig. 3.36d). Some cases have also had increased bone marrow eosinophils.

It is evident that this leukaemia results from mutation in a multipotent myeloid stem cell, often in a myelodysplastic setting.

Immunophenotype The characteristic immunophenotype is expression of CD13, CD33, CD9, HLA-DR

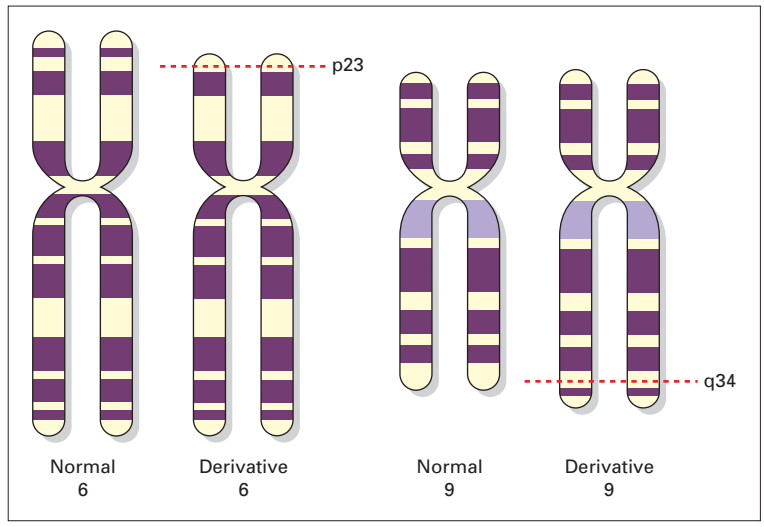
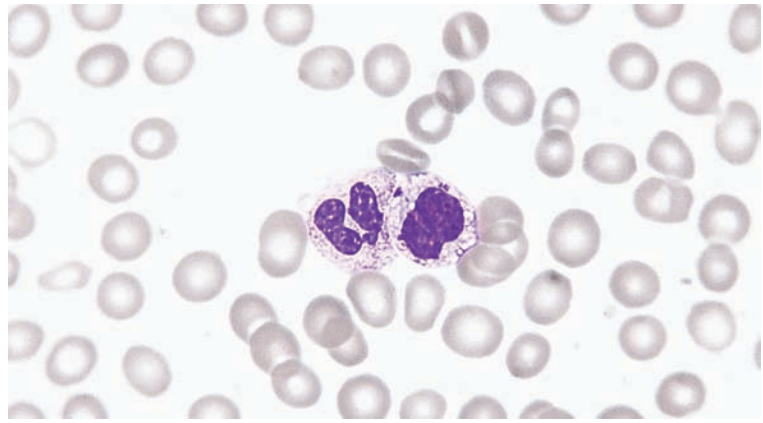
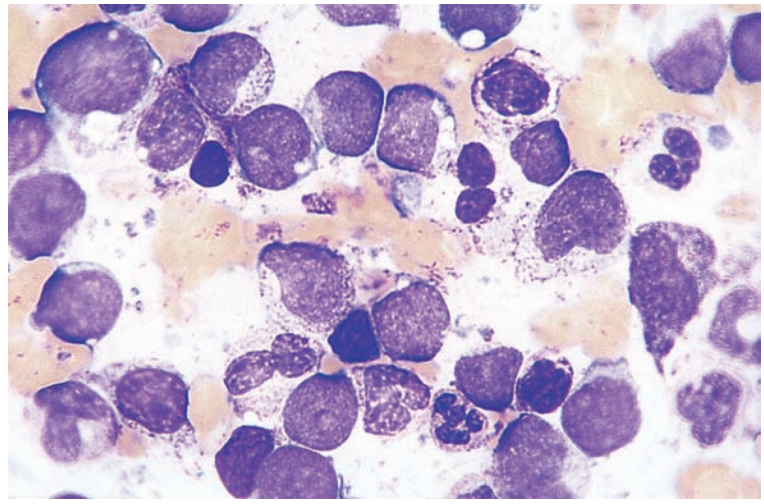


Fig. 3.35 Diagrammatic representation of t(6;9)(p23;q34). (Modified from reference 2.)



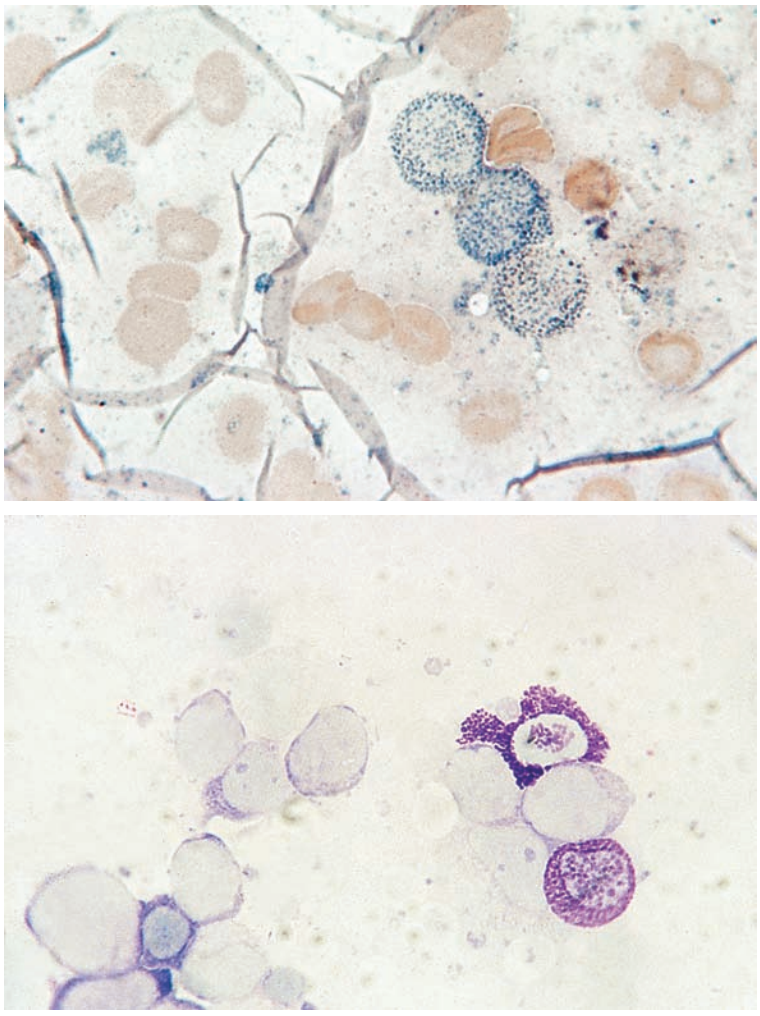
(a)



(b)

Fig. 3.36 PB and BM films from a patient with FAB M2Baso AML associated with t(6;9)(p23;q34). (a) PB film showing abnormal basophils. MGG ×100. (b) BM film showing neutrophilic and basophilic differentiation. MGG ×100.

Continued



(c)

(d)

Fig. 3.36 (*Continued*) (c) BM film showing positive reaction for chloroacetate esterase (CAE). CAE ×100. (d) BM film showing metachromatic staining with toluidine blue. Toluidine blue ×100. (By courtesy of Dr David Swirsky, Leeds.)

and CD34 [240] and often also of CD15, CD34 and CD117 [6]. In contrast to AML in general, TdT is often expressed [6,240].

Cytogenetic and molecular genetic features The t(6;9)(p23;q34) rearrangement is shown in Fig. 3.37. The molecular mechanism of leukaemogenesis is a head-to-tail fusion of part of the *DEK* gene at 6p23 with part of the *NUP214* gene at 9q34.1 (previously known as *CAN*) to form a fusion gene, *DEK-NUP214* [241]. *NUP214* encodes a nucleoporin, i.e. a protein belonging to the nuclear pore complex [242], while *DEK* encodes a DNA-binding nuclear protein. *DEK-NUP214* is detectable by FISH and by RT-PCR, the latter providing a potential target for monitoring of MRD. A *FLT3*-ITD is found as a second genetic event in a large proportion of patients [152,157,240] whereas *FLT3*-TKD mutation is rare [157].

The commonest secondary cytogenetic abnormalities are trisomy 8 and trisomy 13.

Acute myeloid leukaemia with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); RPN1-EVI1

Inversions (Fig. 3.38) and translocations (Fig. 3.39) with 3q21 and 3q26.2 breakpoints are found in no more than 2% of haematological neoplasms, including about 1% of cases of AML [17,243]. This group of disorders is sometimes referred to as the

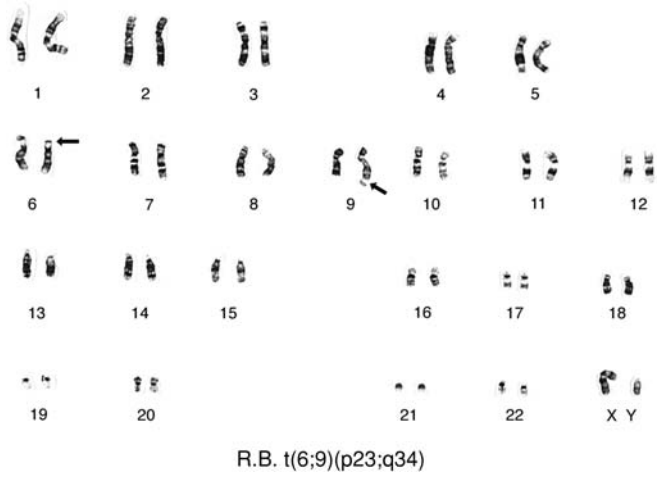


Fig. 3.37 Karyogram of the patient with FAB M2Baso and t(6;9)(p23;q34) whose PB and BM films are shown in Fig. 3.36. (By courtesy of Dr David Swirsky and Miss Julie Bungey, London.)

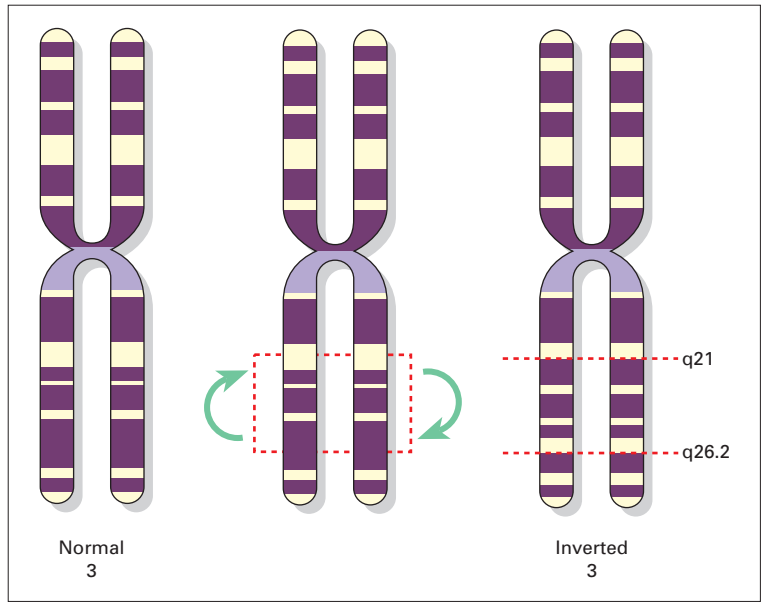


Fig. 3.38 A diagrammatic representation of inv(3)(q21q26.2); this is an example of a paracentric inversion. (Modified from reference 2.)

3q21q26 syndrome. In addition to the association with AML there is an association with MDS and with myeloid blast crisis of Philadelphia (Ph)-positive chronic granulocytic leukaemia (CGL).

Clinical and haematological features 3q21q26.2 abnormalities are associated with both *de novo* and therapy-related AML [212,243]. Only the *de novo* cases fall into this WHO category. Cases occur throughout adult life with the mean age being somewhat younger than that of AML in general [244]. There is a female preponderance [244]. An unusual clinical feature, described in six patients, each of whom also had monosomy 7 (and also observed in another patient with t(3;12)(q26;p12) without monosomy 7), is presentation with diabetes insipidus 1–3 months before diagnosis of AML [245,246]. The AML may be of any FAB category with the exception of M3 AML but cases of M7 AML are over-represented [178,243]. This subtype

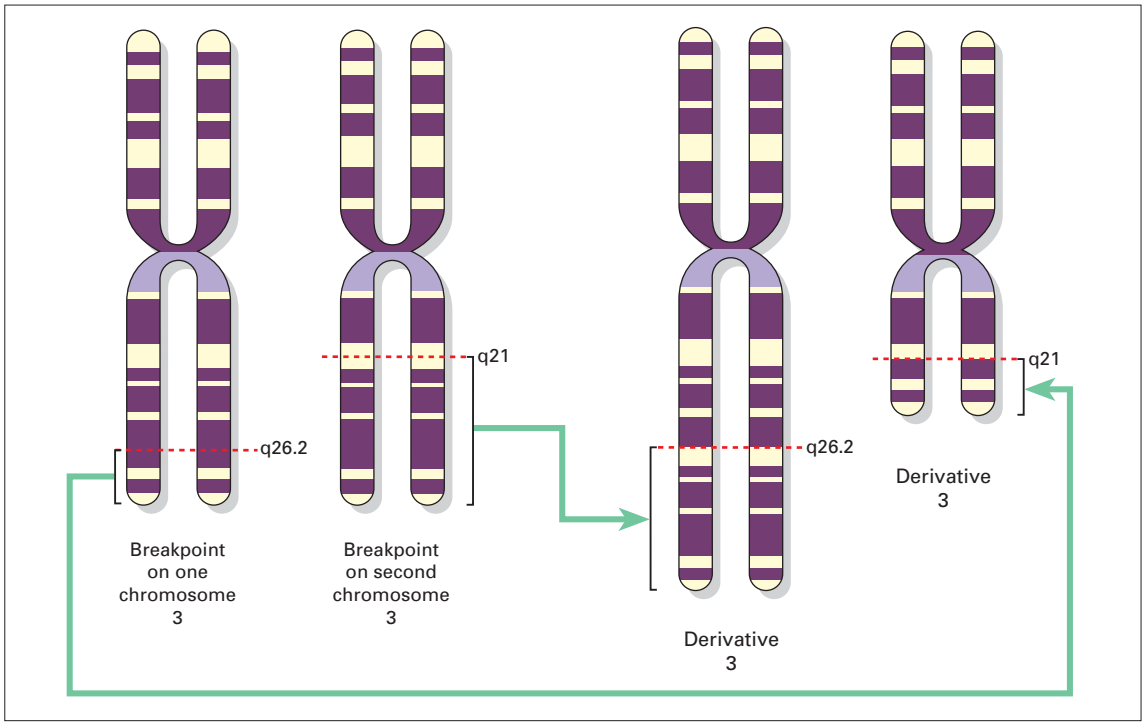


Fig. 3.39 A diagrammatic representation of t(3;3)(q21;q26.2).

of AML is unusual in that the platelet count is normal in about a third of cases and is elevated in some patients. Trilineage myelodysplasia is common. Dyserythropoietic features are non-specific but may include the presence of ring sideroblasts. The main dysplastic features seen in granulocytes are hypogranularity and the acquired Pelger–Huët anomaly. Auer rods are not a feature. Eosinophils and basophils are sometimes increased. Megakaryocytes are often increased in number as well as being dysplastic; both micromegakaryocytes and other dysplastic forms (e.g. multinucleated or large non-lobulated megakaryocytes) are seen (Figs 3.40 and 3.41) and there may be giant or hypogranular platelets [243,247]. There is sometimes associated bone marrow fibrosis.

The associated trilineage myelodysplasia indicates that this subtype of leukaemia results from a mutation in a multipotent stem cell. The prognosis is abysmal with only a quarter to a half of patients achieving a complete remission and the median survival being less than a year [27,47,244]. The 5-year survival is low [18,26,27]. A series of 65 patients entered into MRC trials had a 10-year survival of only 3% [29]. Poor prognosis is not confined to patients with monosomy 7 or a complex karyotype [244].

Immunophenotype No specific immunophenotypic features have been recognized. There is usually expression of CD13, CD33 and CD34 and sometimes of CD7 or megakaryocyte markers such as CD41 or CD61 [6].

Cytogenetic and molecular genetic features The most common cytogenetic abnormality is inv(3)(q21q26.2) (Fig. 3.42) with about a fifth of cases having t(3;3)(q21;q26) (Fig. 3.43) and occasional patients having ins(2;3)(p21;q21q26), ins(3;3)(q23–26;q21q26), ins(5;3)(q14;q21q26) and ins(6;3)(q23;q21q26) [244,248].

The commonest secondary cytogenetic abnormalities are monosomy 7 (which occurs in as many

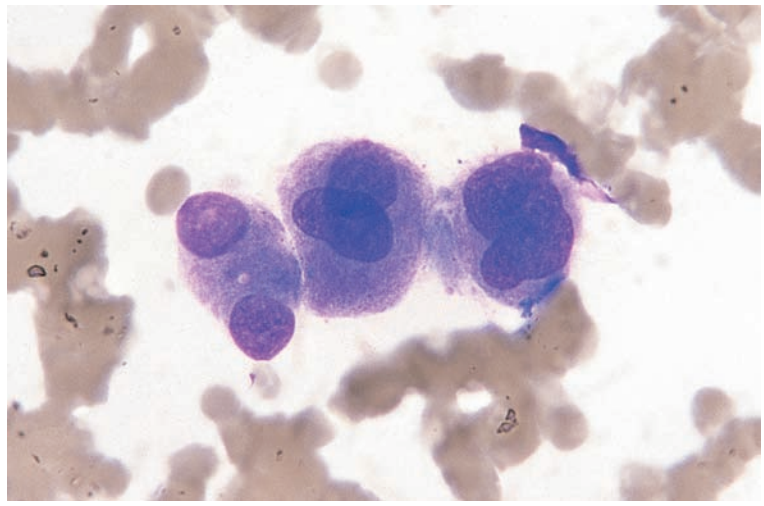


Fig. 3.40 BM film from a patient with inv(3)(q21q26.2) showing increased numbers of dysplastic megakaryocytes. MGG ×100. (By courtesy of Dr Guy Lucas, Manchester.)

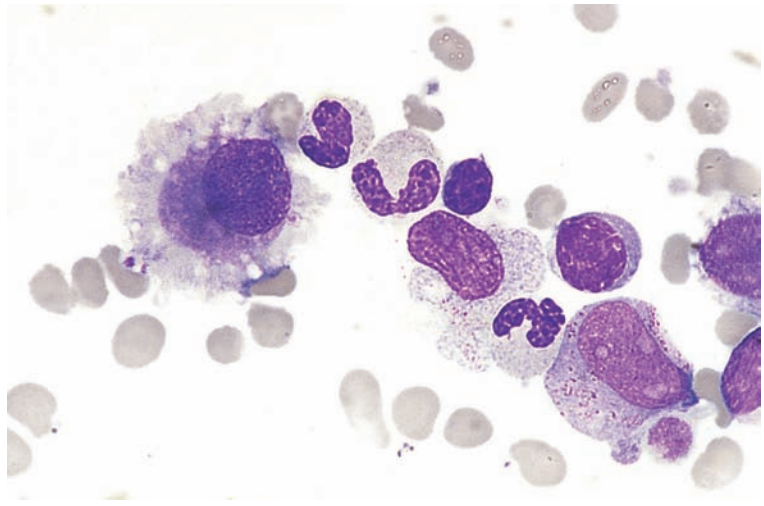


Fig. 3.41 BM film from a patient with t(3;3)(q21;q26.2) showing hypogranular neutrophils and a hypolobulated megakaryocyte. MGG ×100. (By courtesy of the United Kingdom Cancer Cytogenetics Study Group [243].)

as half of patients), trisomy 8, a complex karyotype and del(5q) [210,243,244].

The molecular mechanism of leukaemogenesis appears to be dysregulation of the *EVI1* (ecotropic virus integration 1) gene at 3q26.2 when it is brought into proximity to the enhancer of the ribophorin gene (*RPN1*) at 3q21 [249]. Occasionally there is rearrangement and truncation of *EVI1* [250] but this is not usual [249]. *EVI1* is a transcription factor gene, which is expressed in the kidney and the ovary and in other tissues during embryogenesis but is not expressed in normal haemopoietic cells [249]; it is expressed not only in this subtype of AML but also in some other cases of AML and prognostically adverse subtypes of MDS lacking any cytogenetic abnormality of chromosome 3 [251]. *NRAS* mutations are more common than in AML in general, being found in 27% of patients in one study, in which they did not influence prognosis [83]. *NRAS* mutations were particularly common in patients with t(3;5) in one study, being found in 3 of 8 patients [82] but in another a mutation was found

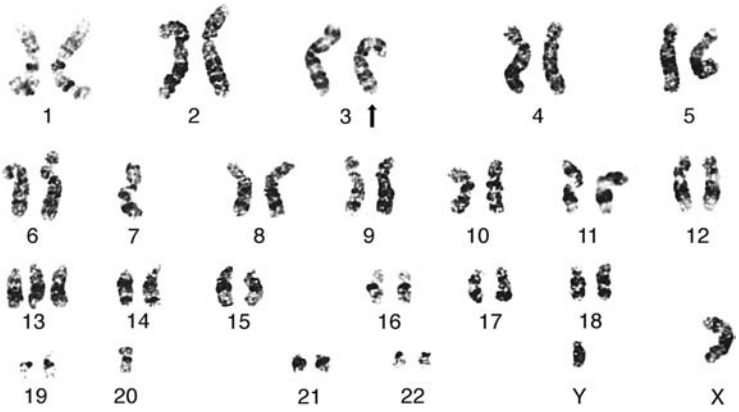


Fig. 3.42 Karyogram showing inv(3)(q21q26.2). (By courtesy of Professor Lorna Secker-Walker.)

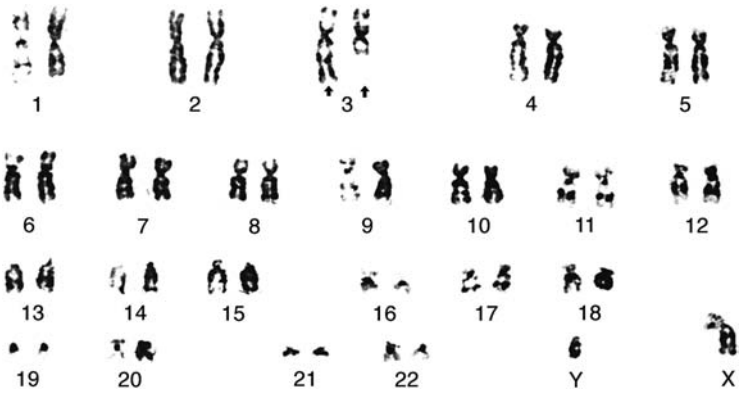


Fig. 3.43 Karyogram showing t(3;3)(q21;q26.2). (By courtesy of Professor Lorna Secker-Walker.)

in none of 14 patients [83]. *FLT3*-ITD is less common than in AML in general, only 13% in one series [244].

This and other rearrangements of 3q26 can be detected by dual-colour, break-apart FISH [248]. Two probe pairs are necessary to cover two breakpoint cluster regions.

Acute myeloid leukaemia with other 3q21 or 3q26 rearrangements

Cases with either a 3q21 or a 3q26 breakpoint but not both share some features with the 3q21q26 syndrome, but there are also some differences; the molecular mechanism of leukaemogenesis is likely to be different and these cases are not assigned to this WHO category. They include t(1;3)(p36;q21) [243,252], t(3;5)(q21;q31) [243,253], t(3;6)(q21;p21) [254], t(3;12)(q26;p13) [243,255], t(3;21)(q26.2;q22) with *RUNX1-EVI1* (which is usually t-AML) [243,256,257] and del(3)(q12q21) [247] (see Table 3.7).

Acute leukaemia (megakaryoblastic) with t(1;22)(p13;q13); RBM15-MKL1

AML associated with t(1;22)(p13;q13) represents less than 1% of cases of AML. This translocation is associated with acute megakaryoblastic leukaemia occurring predominantly in infants and young children. Rarely it occurs in adults [258]. There is a female preponderance.

Clinical and haematological features The median age of reported cases is under 6 months [178,259,260]. Identical twins showing concordance for this subtype of AML have been described suggesting a possible intrauterine origin of the leukaemia [261], and one congenital case has been reported [231]. There may be a female preponderance [258].

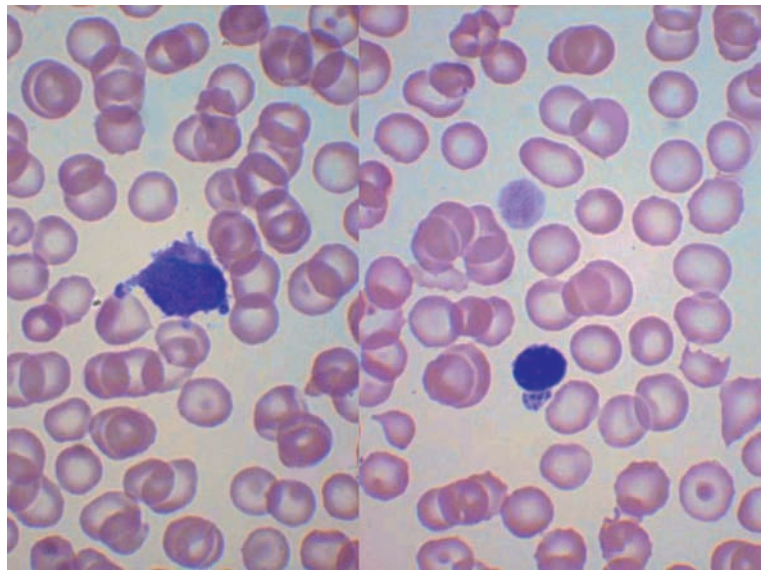


Fig. 3.44 Composite photograph of PB film of an infant with acute megakaryoblastic leukaemia associated with t(1;22)(p13;q13) showing a giant platelet and two megakaryoblasts with granular blebbed cytoplasm. MGG ×100.

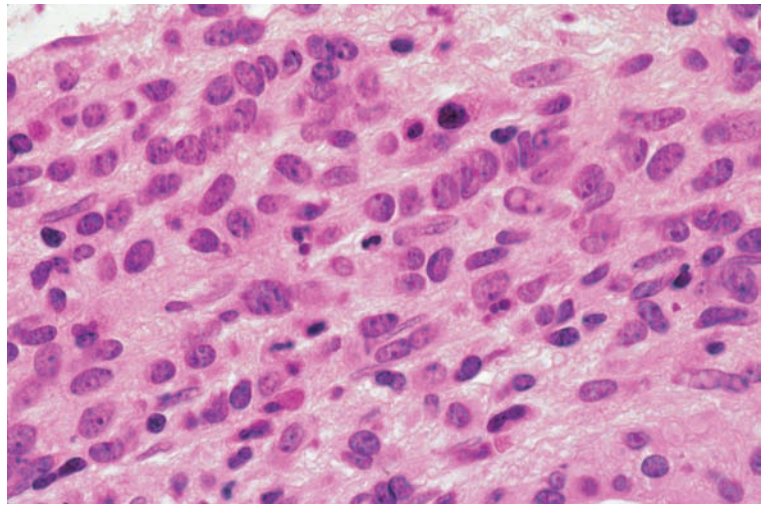


Fig. 3.45 Trephine biopsy section from a patient with FAB M7 AML associated with t(1;22)(p13;q13). Haematoxylin and eosin (H&E) ×100. (By courtesy of Dr Richard D. Brunning, Minnesota.)

Hepatomegaly and splenomegaly are common. The bone marrow blast percentage is low with a series of 10 patients having a median of 22% blasts [260]; in 4 of the 10 patients, blasts were less than 20%. The blast cells may show characteristic features of megakaryoblasts (Fig. 3.44) or may appear morphologically undifferentiated. There is dysmegakaryopoiesis with some micromegakaryocytes [178]. Bone marrow fibrosis can occur (Fig. 3.45). Prognosis is intermediate with a reported remission rate of 55% and 4-year survival of approximately 30% [47].

Immunophenotype There is expression of platelet glycoproteins such as CD41 and CD61 (less often CD42b) [6]. CD13 and CD33 may be expressed but CD34 and HLA-DR are usually negative [6]. CD45 expression may be weak and initial misdiagnosis as a non-haemopoietic tumour has been common among reported cases [210].

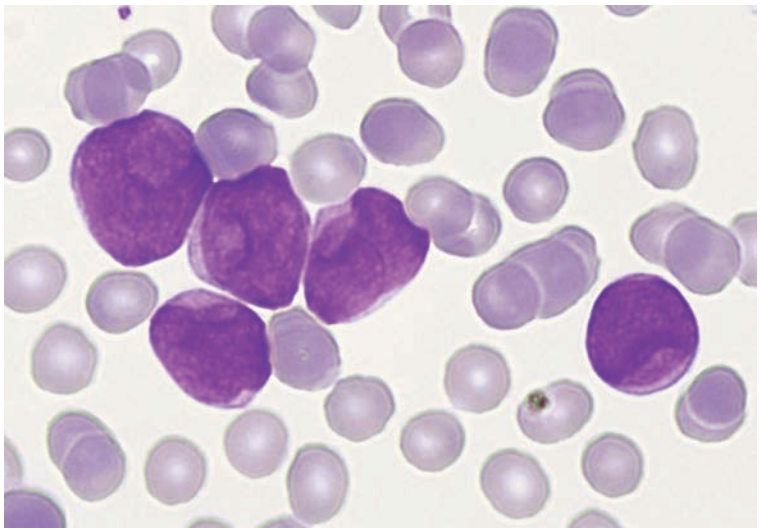


Fig. 3.46 PB film from a patient with AML with a normal karyotype who had both an *NPM1* mutation and *FLT3*-ITD showing blast cells with cup-shaped nuclei. MGG ×100. (By courtesy of Dr Safia Jalal and Dr Mike Leach, Glasgow.)

Cytogenetic and molecular genetic features Hyperdiploidy is a common association with extra chromosomes often including an extra der(1) and extra copies of chromosomes 2, 6, 7, 10, 19 and 21 [47,210,259, 262,263]. The molecular mechanism is formation of an *RBM15-MKL1* fusion gene (previously known as *OTT-MAL*) on chromosome 1 [264,265]; the reciprocal *MKL1-RBM15* fusion gene is not transcribed in all patients. The fusion gene has also been detected in association with a complex variant translocation, with a der(1)t(1;22)(p13;q13), and in a patient with a normal karyotype [178,258].

Acute myeloid leukaemia with NPM1 mutation

Mutations of *NPM1* at 5q35 have been detected in over a third of patients with *de novo* AML, and less frequently in t-AML [266–268]. In those with a normal karyotype the frequency in different series of patients ranges from 46% to 62% [269]. The *NPM1* gene encodes nucleophosmin, a phosphoprotein that shuttles between the nucleus and cytoplasm, and the mutation leads to cytoplasmic rather than nuclear (mainly nucleolar) localization of the protein. The 2008 WHO classification includes a provisional category for AML with *NPM1* mutation in the absence of the recurrent genetic abnormalities described above.

Clinical and haematological features These mutations are more common in women than in men [267,268,270] and in adults than in children. The majority of cases have evidence that the mutation occurs in a multipotent haemopoietic stem cell. There is an association with all FAB subtypes with the exception of M3 but there is a particular association with FAB types M4 and M5 [266,270,271] and with gingival hyperplasia [267], lymphadenopathy [267] and skin infiltration. The WBC [267,270–272], bone marrow blast percentage [268] and platelet count [268] tend to be higher than in patients without an *NPM1* mutation. Multilineage dysplasia may be present. An association with blast cells with invaginated nuclei (cup-shaped nuclei) (Fig. 3.46) has been observed [273,274] but this finding is not specific and is also associated with *FLT3* mutation [274].

Immunophenotype There is expression of CD13, CD33 and MPO and often of CD14, CD11b and CD68R [6]. The stem cell markers, CD34 and CD133, are characteristically negative [270]. HLA-DR is expressed in about two thirds of patients and CD7 is expressed in a minority. Cytoplasmic rather than nuclear expression of NPM1, detectable by immunohistochemistry [275] or flow cytometry [276], acts as a surrogate marker for an *NPM1* mutation.

Cytogenetic and molecular genetic features The mutations (small duplications or insertions) all lead to a

frame-shift in the region of the gene that encodes the C-terminal protein. Mutations can be detected by multiplex PCR-based fragment length analysis [277]. The karyotype is characteristically normal and when karyotypic abnormalities are present they are usually secondary rather than primary events.

There is a high frequency of associated *FLT3*-ITD, which appears to be a second event [268]. There is an inverse association with *CEBPA* mutations, *NRAS* mutations, *MLL* partial tandem duplication (*MLL*-PTD), t(8;21), t(15;17), inv(16) and complex karyotypes [268,271,272]. Only occasionally is an *NPM1* mutation found in patients with t(8;21) or inv(16) [268] and such cases are categorized according to the cytogenetic abnormality. There is a characteristic gene expression profile [272]. In one study prognosis did not differ between patients with a normal karyotype with or without an *NPM1* mutation [271]. However, in most studies the presence of an *NPM1* mutation in the absence of a *FLT3*-ITD, in those with normal cytogenetic analysis or in the intermediate prognosis cytogenetic group, correlates with a higher remission rate [267,270], longer event-free survival [267,268,270,272] and longer overall survival [267,268,272]. Patients with an *NPM1* mutation but without *FLT3*-ITD mutation or adverse cytogenetic abnormalities could reasonably be assigned to the good prognosis category of AML. Five-year survival is about 60% [267].

MRD can be monitored by RQ-PCR; three sets of primers cover 90% of mutations.

Acute myeloid leukaemia with CEBPA mutation

Around 6–15% of cases of *de novo* AML are associated with mutation of *CEBPA*, the gene encoding the myeloid transcription factor, CEBPα (CCAAT/enhancer binding protein α) [278]. Among patients with a normal karyotype, the prevalence in a large series of patients was 8–9% [279]. The 2008 WHO classification includes a provisional category for AML with *CEBPA* mutation in the absence of the recurrent genetic abnormalities described above.

Germ-line heterozygous mutation of this gene is associated with adult-onset AML in a rare familial syndrome [280].

Clinical and haematological features *CEBPA* mutations have been associated particularly with FAB M1 AML but have also been observed in M2, M4 and M5 categories. Extramedullary disease is not common.

Immunophenotype There is expression of myeloid antigens such as CD13, CD33, CD65, CD11b and CD15 [6]. CD34 and HLA-DR are usually expressed and CD7 is expressed in half to three quarters of patients [6].

Cytogenetic and molecular genetic features Mutations may be either an N-terminal dominant negative frame-shift loss of function mutation or a C-terminal mutation that reduces DNA binding [281]. Multiplex PCR-based fragment length analysis, with a false-negative rate of less than 1%, is suitable for routine diagnostic use [279]. *CEBPA* mutations are associated with miscellaneous cytogenetic abnormalities falling into the intermediate prognosis group of AML. There is a particular association of loss of function mutations with del(9q) [281]. *CEBPA*-mutated AML has a specific gene expression profile, which is shared with a group of cases that have *CEBPA* silencing, usually as a result of promoter hypermethylation [282]; coexpression of CD7 is seen in both groups. There is an associated *FLT3*-ITD in a fifth to a third of patients [6]. *CEBPA* mutations are associated with a good prognosis, particularly if there is no coexisting *FLT3*-ITD; those without *FLT3*-ITD or adverse cytogenetic abnormalities could reasonably be assigned to the good prognosis category, together with cases of AML associated with *RUNX1-RUNX1T1*, *PML-RARA*, *CBFB-MYH11* and *NPM1* mutation without *FLT3*-ITD [278].

Approaching 4% of patients with AML and a normal karyotype have mutation of both *CEBPA* and *NPM1* [269].

Acute myeloid leukaemia with myelodysplasia-related changes

Most studies have found multilineage dysplasia to have an adverse prognostic significance in patients with AML [283–287]. For this reason the 2001 WHO classification distinguished such cases from AML, not otherwise specified. Dysplasia had to be present in at least 50% of cells in at least two myeloid lineages. AML with multilineage dysplasia could arise *de novo* or follow MDS. This subtype was found to correlate with older age and unfavourable

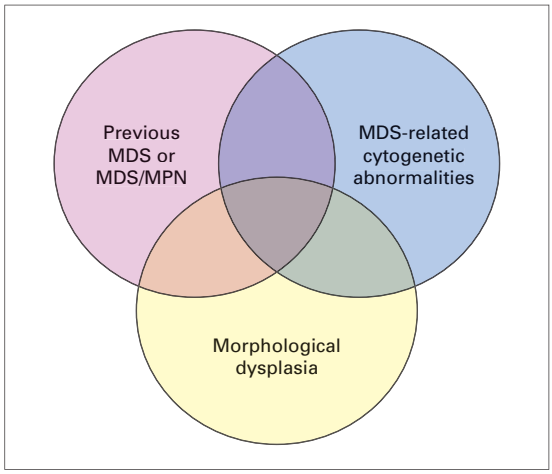


Fig. 3.47 A diagram illustrating that cases may qualify to be classified as myelodysplasia-related AML by meeting one, two or three criteria. MDS/MPN, myelodysplastic/myeloproliferative neoplasm.

karyotypes [285]. The definition of this category was expanded, in the 2008 WHO classification, to include patients with prior MDS or specified adverse karyotypes, whether or not they met the morphological criteria at the time of diagnosis of AML [288] (Fig. 3.47). These criteria have now been validated by the observation that cases defined in this manner (48% of all AML) are associated with a lower remission rate, a shorter progression-free survival and reduced overall survival in comparison with AML, not otherwise specified [289]. The prognostic differences were highly significant. In this group of 48 patients, 41 patients met the morphological criteria, 16 had had previous MDS and 14 had myelodysplasia-related karyotypic abnormalities (in seven instances without meeting morphological criteria for dysplasia) [289].

Clinical and haematological features Patients tend to be elderly and may have had previous MDS or myelodysplastic syndromes/myeloproliferative neoplasms (MDS/MPN). By definition, there is no prior exposure to cytotoxic chemotherapy or irradiation. Pancytopenia is common. Cytological features may be those of any FAB category except FAB M3. FAB M6 is over-represented. Multilineage dysplasia is common but not invariable (Figs 3.48–3.50). Prognosis is generally poor. A group of 797 patients entered into MRC trials had a 10-year survival of 16% [29]. Progression may be less rapid in children with a low blast percentage, e.g. 20–30%, and in adults with prior MDS and relatively low blast counts [288].

Immunophenotype There is expression of myeloid antigens such as CD13 and CD33 [288]. CD34, TdT, CD7 and CD56 are often expressed.

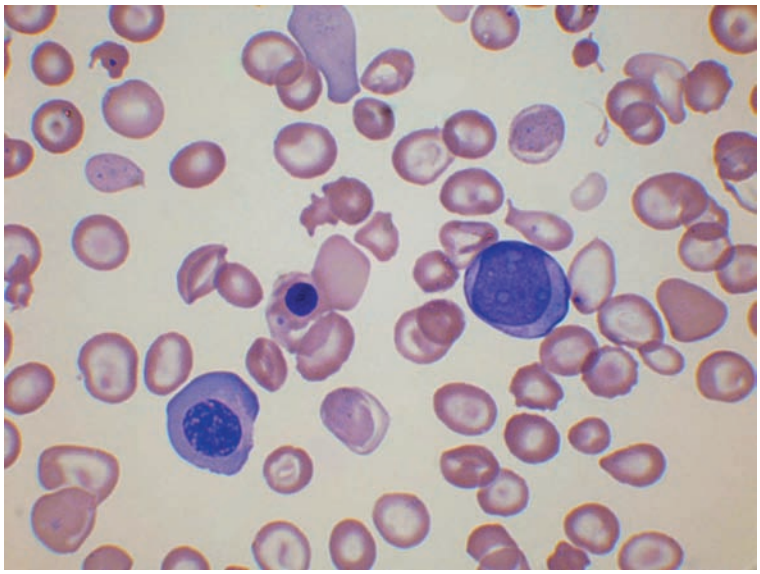


Fig. 3.48 PB film from a patient with myelodysplasia-related AML showing a blast cell and marked anisopoikilocytosis, basophilic stippling, polychromasia and nucleated red blood cells (one of which is megaloblastic). MGG ×100.

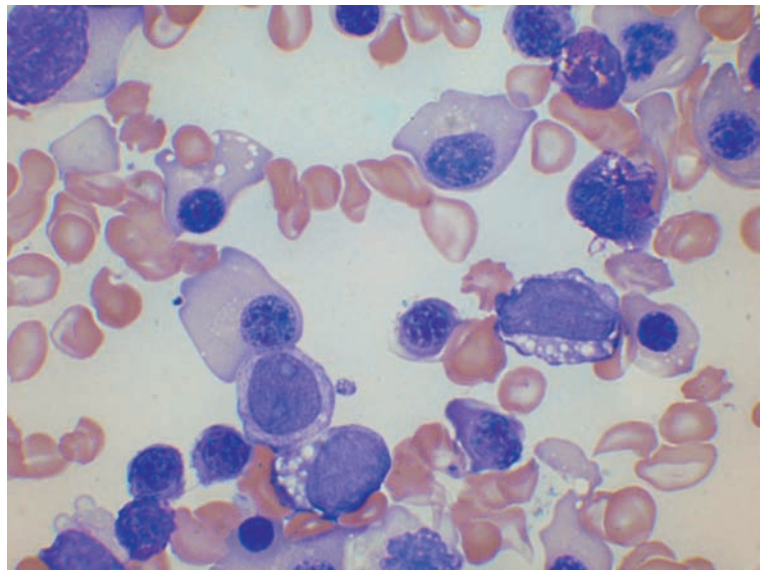


Fig. 3.49 BM film from a patient with myelodysplasia-related AML (same patient as Fig. 3.48) showing two vacuolated blast cells and dyserythropoiesis, including the presence of numerous megaloblasts. MGG ×100.

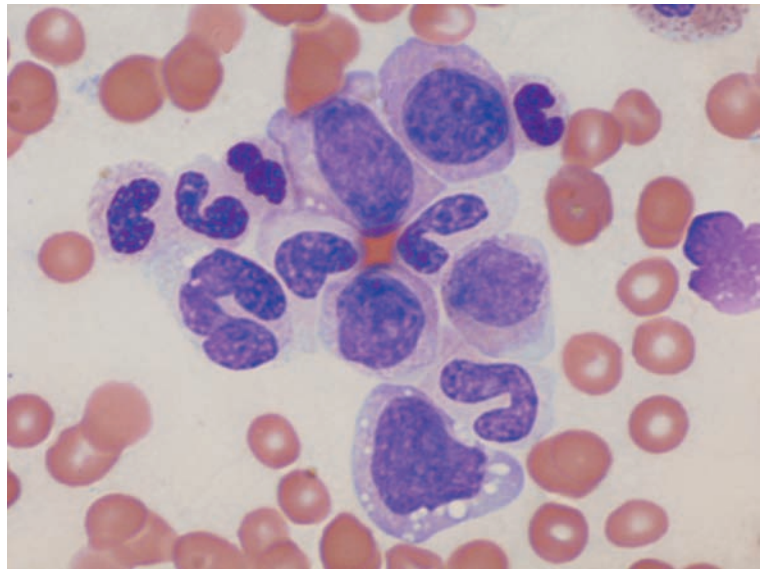


Fig. 3.50 BM film from a patient with myelodysplasia-related AML showing markedly dysplastic granulocytes and precursors. MGG ×100.

Cytogenetic and molecular genetic features By definition, the recurrent cytogenetic/genetic abnormalities that define definitive WHO categories of AML are absent. However, cases with an *NPM1* or *CEBPA* mutation are *not* excluded. *FLT3*-ITD may be present. Cytogenetic abnormalities are often complex and unbalanced and may include abnormalities of chromosomes 5 and 7. The cytogenetic abnormalities that qualify a case for inclusion in this WHO category are shown in Table 3.5.

Therapy-related myeloid neoplasms

Two broad groups of t-AML were recognized in the 2001 WHO classification [290]. In the first type, MDS and AML occurred following exposure to either alkylating agents (e.g. chlorambucil, busulphan,

Complex	Unbalanced	Balanced
Greater than three unrelated abnormalities (none of which would qualify a case to be categorized as AML with recurrent genetic abnormality)	–7 or del(7q) –5 or del(5q) i(17q) or t(17p) –13 or del(13q) del(11q) del(12p) or t(12p) del(9q) idic(X)(q13)	t(11;16)(q23;p13.3)* t(3;21)(q26.2;q22.1)* t(1;3)(p36.3;q21.2) t(2;11)(p21;q23)* t(5;12)(q33;p12) t(5;7)(q33;q11.2) t(5;17)(q33;p13) t(5;10)(q33;q21) t(3;5)(q25;q34)

Table 3.5 Cytogenetic abnormalities that permit categorization of a case with at least 20% of myeloid blast cells in the peripheral blood or the bone marrow as AML with myelodysplasia-related changes [288].

AML, acute myeloid leukaemia; t-AML, therapy-related AML.
* As long as t-AML is excluded.

melphalan, cyclophosphamide, carboplatin, cisplatin, dacarbazine, procarbazine, mitomycin C), nitrosoureas (e.g. carmustine (BCNU) and lomustine (CCNU)) or ionizing radiation. MDS or acute leukaemia, the latter often evolving from MDS, usually occurred 5–10 years after drug exposure. Cases of AML often showed trilineage dysplasia. Auer rods were less common than in *de novo* AML whereas increased basophils, bone marrow hypocellularity and bone marrow fibrosis were more common. The leukaemia could be of any FAB type but was rarely M3 and M6 was over-represented. Cases of this type of t-AML can be difficult to assign to a FAB category. The prognosis is generally poor. Common and uncommon cytogenetic abnormalities in t-AML are shown in Table 3.6.

A second type of therapy-related acute leukaemia was recognized following exposure to topoisomerase II-interactive drugs, both the topoisomerase II inhibitors (epipodophyllotoxins such as etoposide and teniposide) and intercalating topoisomerase II inhibitors such as the anthracyclines (daunorubucin, doxorubicin and epirubicin), mitoxantrone, dactinomycin and dioxypiperazine derivatives such as bimolane. The interval between exposure to the drug and the development of leukaemia was shorter than with the alkylating agents, often only 1–5 years. Although MDS could occur it was less common than when t-AML followed alkylating agents. Characteristic chromosomal abnormalities are shown in Table 3.6. In the case of some of these chromosomal abnormalities, e.g. those with 11q23 breakpoints, quite a large proportion of cases are t-AML whereas in others, such as t(8;21)(q22;q22) and t(15;17)(q22;q12), the therapy-related cases are only a small proportion of total cases. In contrast to acute leukaemia following the alkylating agents, occasional cases following topoisomerase II-interactive drugs have been lymphoblastic (see page 195) or mixed phenotype rather than myeloid; these cases are related to translocations with an 11q23 breakpoint. The prognosis of t-AML following the topoisomerase II-interactive drugs is not necessarily as bad as that of cases following the alkylating agents but appears to be worse than that of *de novo* AML with the same cytogenetic abnormality. The uncommon cases of secondary leukaemia with t(8;21)(q22;q22) and t(15;17)(q22;q12) appear to have a similar complete remission rate to *de novo* cases; although little information is available on the long-term prognosis it appears to be worse than that of *de novo* cases [24,100]. Cases involving the *MLL* gene certainly have a poor long-term prognosis despite an initially high complete remission rate [149,206]. In addition to the difference in cytogenetic abnormalities between disease induced by different types of agent, there are also differences in other molecular abnormalities [296]. For example, *TP53* and *RAS* mutations and *RUNX1* and *MLL* duplications and amplifications are particularly associated with alkylating agent-induced disease [296].

It is suspected that anti-metabolites, e.g. azathioprine and fludarabine, can also cause t-AML [297]. The cytogenetic abnormalities described have been those characteristic of alkylating agent-related t-AML/MDS.

In the 2008 WHO classification, cases of t-AML are no longer categorized according to the putative causative agent and are grouped with therapy-related MDS and therapy-related MDS/MPN [298].

Table 3.6 Cytogenetic abnormalities associated with therapy-related acute leukaemia [22–24,100,149,206,291–295].

Following alkylating agents and nitrosoureas	Following topoisomerase II-interactive drugs
Drugs incriminated	
Chlorambucil, busulphan, cyclophosphamide, melphalan, carmustine (BCNU), lomustine (CCNU)	Etoposide, teniposide, doxorubicin, daunorubicin, epirubicin, mitoxantrone, bimolane, razoxane, dactinomycin
Chromosomal rearrangements	
Complex chromosomal abnormalities, often including –7, 7q–, –5 and 5q–; also 12p–, –17, 17p–, 13q–,–18, 20q–, der(1)t(1;7)(p11;p11) and other unbalanced translocations leading to loss of part of 5q or 7q and/or dup of (1q): t(1;3)(p36;p21) [295] inv(3)(q21q26) t(3;3)(q21;q26) t(6;9)(p23;q34.3) t(8;16)(p11;p13)	Chromosomal rearrangements with an 11q23 breakpoint (*MLL* often shown to be rearranged): t(1;11)(p32;q23)* t(1;11)(q21;q23) t(3;11)(p21;q23) t(3;11)(q25;q23) t(3;11)(q28;q23) t(4;11)(q21;q23)† t(5;11)(q35;q23)* t(6;11)(q27;q23) t(9;11)(p21;q23) t(10;11)(p11;q23) t(10;11)(p13;q23) inv(11)(p14q23) t(11;11)(p13-15;q23) t(11;16)(q23;p13)† t(11;17)(q23;p13) cryptic t(11;17)(q23;q25) t(11;19)(q23;p13.3)† t(11;19)(q23;p13.1) t(11;21)(q23;q22) Chromosomal rearrangements with a 21q22 breakpoint (*RUNX1* often shown to be rearranged): t(1;21)(p36;q22) t(3;21)(q26;q22) t(7;21)(q31;q22) t(8;21)(q22;q22) t(16;21)(q24;q22) Chromosomal rearrangements with an 11p15 breakpoint (*NUP98* often shown to be rearranged): t(1;11)(q23;p15) t(2;11)(q35;p15) t(7;11)(p15;p15) [291] t(10;11)(q22~23;p15) [291] inv(11)(p15q22 or q23) t(11;17)(p15;q21) t(11;20)(p15;q11) Other: t(6;9)(p23;q34.3) t(8;16)(p11;p13) t(9;22)(q34;q11)‡ [291] t(15;17)(q22;q12) [100] inv(16)(p13q22) [100] t(16;16)(p13;q22) [100]

* Therapy-related acute lymphoblastic leukaemia (ALL) [293].
† Including therapy-related ALL [293].
‡ Therapy-related acute myeloid leukaemia, ALL or chronic granulocytic leukaemia.

Clinical and haematological features Patients include children as well as adults. Relevant exposure to leukaemogenic agents may have been for a haematological or non-haematological neoplasm or for a non-neoplastic condition (e.g. immunosuppressive treatment for disease or in relation to allogeneic transplantation of various types). Multilineage dysplasia is common, particularly following alkylating agents. Cytological features may be those of any of the FAB categories (including FAB M3 AML). An increased basophil count, hypocellularity and bone marrow fibrosis are all more common than in *de novo* AML or MDS. The prognosis is poor, particularly with t-MDS/t-AML typical of alkylating agent exposure.

Immunophenotype The immunophenotype is heterogeneous and non-specific but aberrant expression of CD7 or CD56 is common.

Cytogenetic and molecular genetic features There is a very high frequency of cytogenetic aberrations, the most characteristic being shown in Table 3.6.

Acute myeloid leukaemia, not otherwise specified

The WHO category of acute myeloid leukaemia, not otherwise specified (AML, NOS) [299] is a default category for cases that do not meet the criteria for the definitive and provisional subtypes of AML that have been discussed above. Myeloid sarcoma and myeloid neoplasms related to Down syndrome are also excluded. Many of the subtypes of this category are similar to FAB categories, except that a lower blast percentage is accepted for diagnosis and many of the more specific entities have been removed. Other subtypes were not specifically recognized in the FAB classification.

Some patients designated AML, NOS have recurrent cytogenetic abnormalities other than those that the WHO classification recognizes as defining specific categories. These may, nevertheless, be discrete entities, and some of them, e.g. AML with t(8;16)(p11.2;p13.3), are discussed below.

Acute myeloid leukaemia with minimal differentiation

Acute myeloid leukaemia with minimal differentiation resembles the FAB M0 category (see page 12) except that cases meeting the criteria for the specific entities defined above are excluded and a blast count of 20% in either the blood or bone marrow is sufficient for the diagnosis.

Acute myeloid leukaemia without maturation

Acute myeloid leukaemia without maturation resembles the FAB M1 category (see page 16) except that cases meeting the criteria for the specific entities defined above are excluded and a blast count of 20% in either the blood or bone marrow is sufficient for the diagnosis.

Acute myeloid leukaemia with maturation

Acute myeloid leukaemia with maturation resembles the FAB M2 category (see page 19) except that cases meeting the criteria for the specific entities defined above, e.g. cases with t(8;21)(q22;q22), are excluded. A blast count of 20% in either the blood or bone marrow is sufficient for the diagnosis whereas the FAB M2 category requires 30% blast cells.

Acute myelomonocytic leukaemia

Acute myelomonocytic leukaemia resembles the FAB M4 category (see page 27) except that cases meeting the criteria for the specific entities defined above, e.g. many cases with inv(16)/t(16;16) or with t(9;11) or other rearrangement of *MLL*, are excluded. A blast count (including promonocytes) of 20% in either the blood or bone marrow is sufficient for the diagnosis.

Acute monoblastic/monocytic leukaemia

Acute monoblastic/monocytic leukaemia resembles the FAB M5 category (see page 30) except that cases meeting the criteria for the specific entities defined above, e.g. many cases with t(9;11) or other rearrangement of *MLL*, are excluded. A blast count (including promonocytes) of 20% in either the blood or bone marrow is sufficient for the diagnosis. The WHO classification recognizes the existence of monoblasts that lack non-specific esterase activity but can be recognized by immunophenotyping (which would fall into the FAB M0 category).

Acute erythroid leukaemia

Within the group designated acute erythroid leukaemia the WHO classification recognizes an erythroleukaemia (erythroid/myeloid leukaemia), which is similar to the FAB category of M6 AML,

and a pure erythroid leukaemia, in which more than 80% of bone marrow cells are erythroid and there is no evidence of a significant myeloblastic component. The latter, a rare condition, was recognized, in 1923, by Giovanni Di Guglielmo who suggested the name 'acute erythremic myelosis' [300]. The designation 'M6 variant AML' has also been proposed [301]. Pure erythroid leukaemia is characterized by the dominance of cells that are either medium or large erythroid cells, recognizable by conventional cytological features, or blast-like cells that can be shown to be erythroid by immunophenotyping or by specialized techniques, such as transmission electron microscopy. Antigens that may be expressed include glycophorin and haemoglobin A by the more mature cells and carbonic anhydrase, the Gerbich blood group antigen and CD36 by less mature cells. CD36 is not lineage specific [299]. CD34 and HLA-DR are often negative but CD117 may be positive.

More than half the patients who would otherwise meet the WHO criteria for erythroid/myeloid or pure erythroid leukaemia have myelodysplasia-related features and are therefore assigned to that WHO category [302].

Acute megakaryoblastic leukaemia

Acute megakaryoblastic leukaemia resembles the FAB M7 category (see page 40) except that it is more precisely defined (at least 50% of blasts are megakaryoblasts) and cases meeting the criteria for the specific entities defined above, e.g. acute megakaryoblastic leukaemia associated with t(1;22), are excluded. Babies and infants with myeloid neoplasms related to Down syndrome (see below) are also excluded. A blast count of 20% in either the blood or bone marrow is sufficient for the diagnosis. Some but not all cases have prominent fibrosis.

Acute basophilic leukaemia

Acute basophilic leukaemia was not specifically identified in the FAB classification. Many cases would have fallen into the M2 category. Differentiation is primarily to basophils but there may be blasts with granules characteristic of mast cells as well as basophil-type granules. Clinical features may include those resulting from histamine excess. Blasts of basophil lineage may be packed with basophilic granules (somewhat resembling acute promyelocytic leukaemia) (Fig. 3.51) or may have more sparse granules and cytoplasmic vacuolation (Fig. 3.52). They may have blocks or lakes of PAS-positive material. Immunophenotyping may show expression of CD11b, CD123, CD203c and often CD9 in addition to CD13 and CD133 [299]. Basophil (or mast cell) differentiation can be confirmed by metachromatic staining with toluidine blue (Fig. 3.53). On immunophenotyping, blast cells express myeloid antigens but are also often positive for CD9 and CD25 [42]. CD203c is expressed but it is also expressed by mast cells. The presence of t(9;22) and *BCR-ABL1* should be excluded.

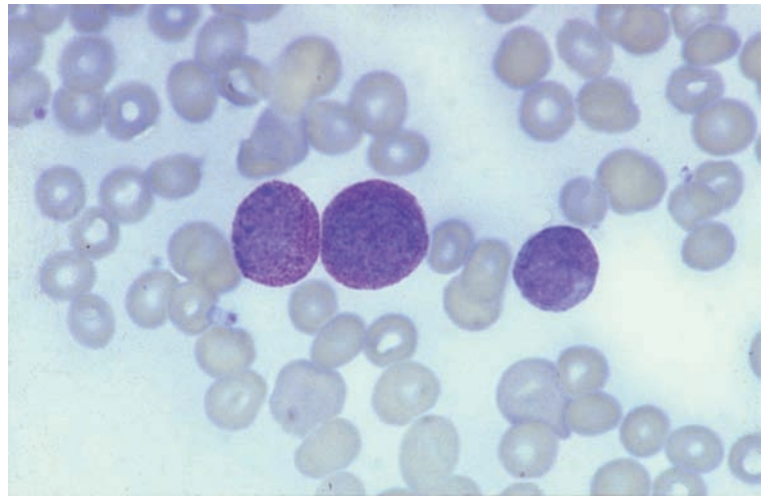


Fig. 3.51 PB film in acute basophilic leukaemia showing heavily granulated blast cells. MGG ×100.

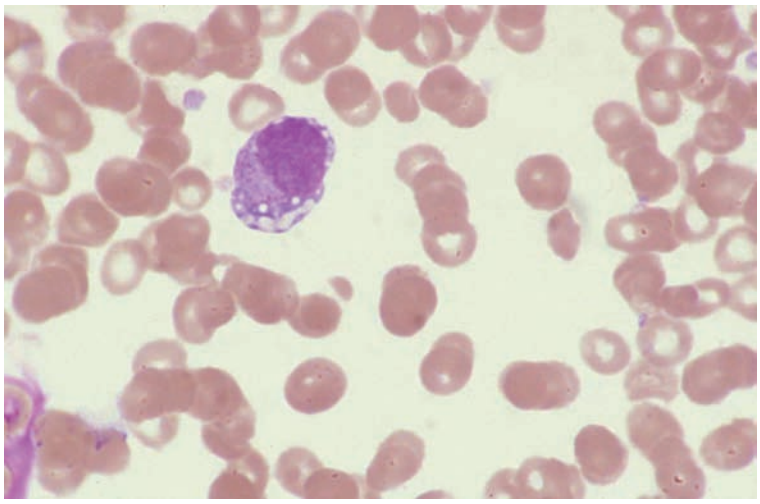


Fig. 3.52 PB film in acute basophilic leukaemia showing a vacuolated blast cell with scanty granules. MGG ×100.

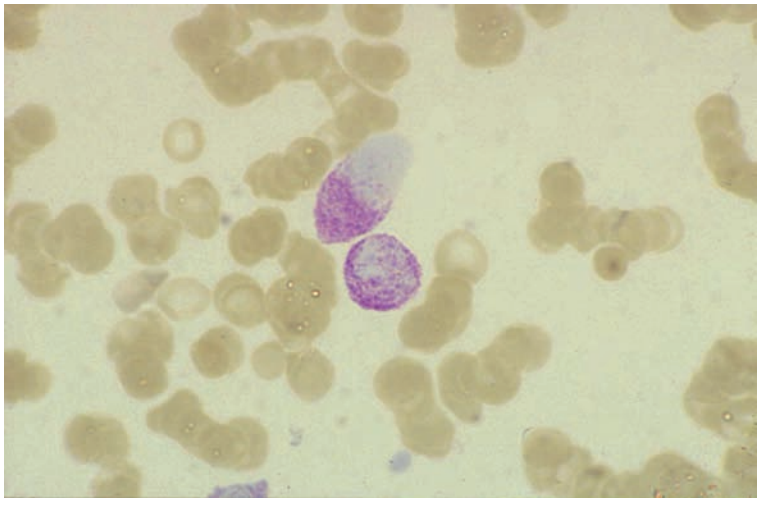


Fig. 3.53 PB film in acute basophilic leukaemia showing blast cells that stain metachromatically with toluidine blue. Toluidine blue ×100.

Acute panmyelosis with myelofibrosis

This condition, which was introduced into the 2001 WHO classification, was not recognized in the FAB classification. It comprises 1–2% of cases of AML [303]. These patients usually have pancytopenia with a leucoerythroblastic blood film, mild poikilocytosis, absent teardrop cells and few circulating blasts. The bone marrow is hypercellular and shows increased cells of all myeloid lineages with a disproportionate increase in immature cells; there is increased reticulin deposition and sometimes collagen fibrosis (Fig. 3.54). The blast count is usually relatively low, e.g. 20–25% [299]. Blast cells are a variable mixture of myeloblasts or monoblasts, proerythroblasts and megakaryoblasts (Fig. 3.55). There may be prominent inflammatory changes including lymphoid nodules, increased plasma cells and increased vascularity [304]. The condition previously described as acute myelofibrosis includes some cases that meet the WHO criteria for acute panmyelosis with myelofibrosis but other cases of acute myelofibrosis represent acute megakaryoblastic leukaemia with fibrosis [305].

Myeloid sarcoma

Myeloid sarcomas are solid extramedullary tumours that can be can be composed predominantly of cells showing granulocytic differentiation (granulocytic

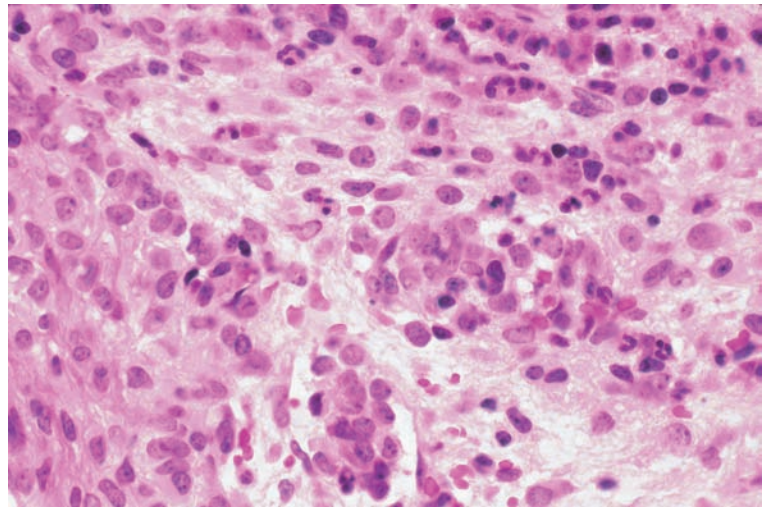
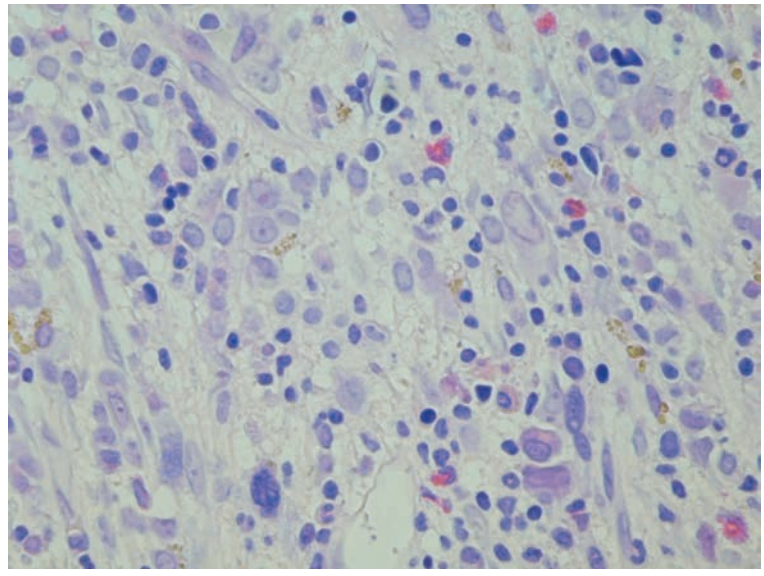
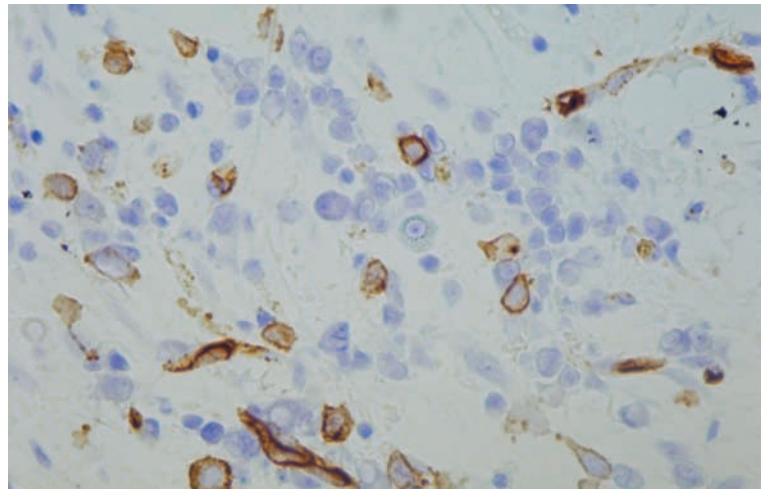


Fig. 3.54 Trephine biopsy section from a patient with acute panmyelosis. H&E ×100. (By courtesy of Dr Richard D. Brunning.)



(a)

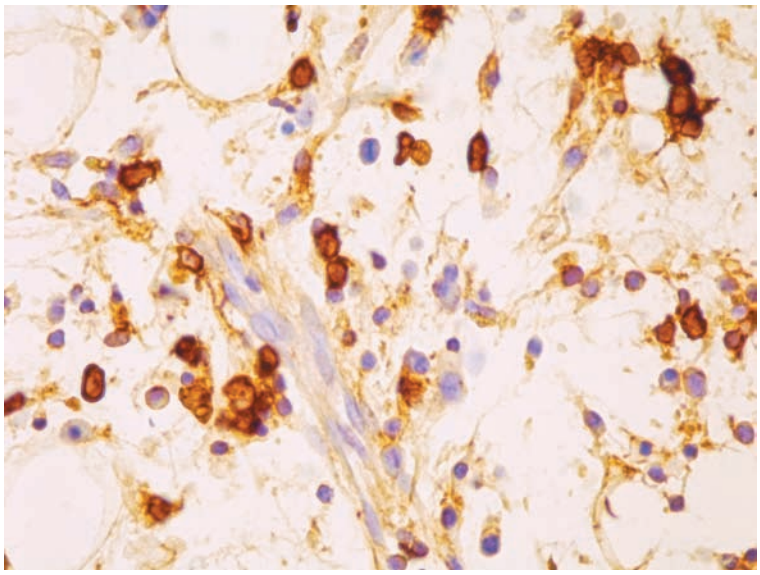


(b)

Fig. 3.55 Trephine biopsy sections from a patient with acute panmyelosis. (a) Blast cells and maturing erythroid and granulocytic cells irregularly arranged in a loose fibrous stroma; there is one dysplastic megakaryocyte and haemosiderin is apparent. H&E ×60. (b) Blast cells and the endothelial cells of small blood vessels are highlighted by CD34. Immunoperoxidase ×60.

Continued

(c)



(d)

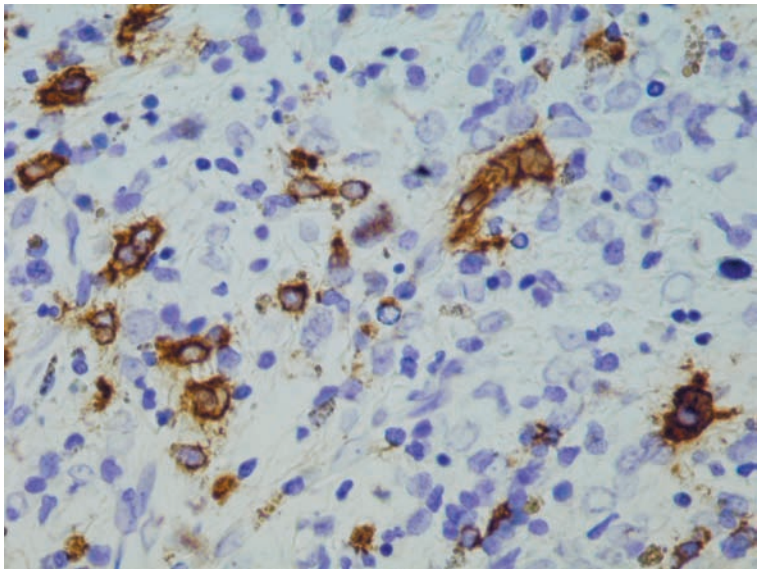
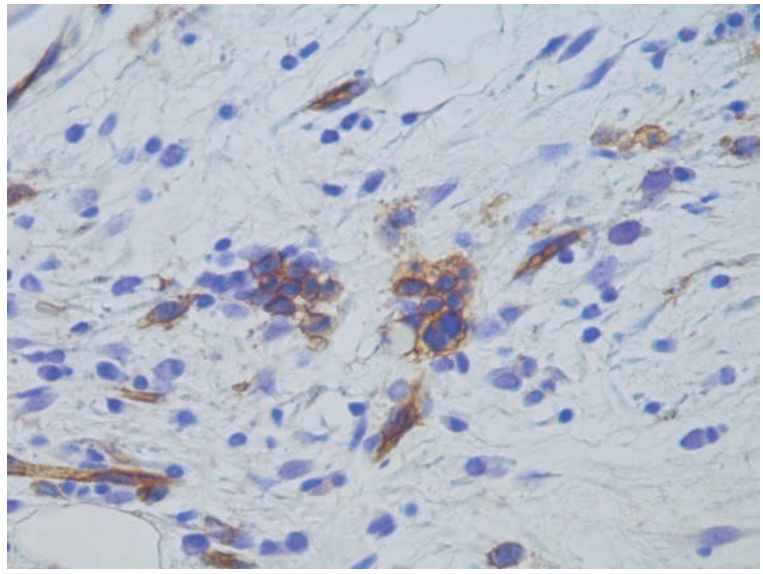


Fig. 3.55 (*Continued*) (c) Myeloblasts are highlighted by their lysozyme content. Immunoperoxidase ×60. (d) Megakaryoblasts and dysplastic megakaryocytes are highlighted by detection of CD61. Immunoperoxidase ×60.

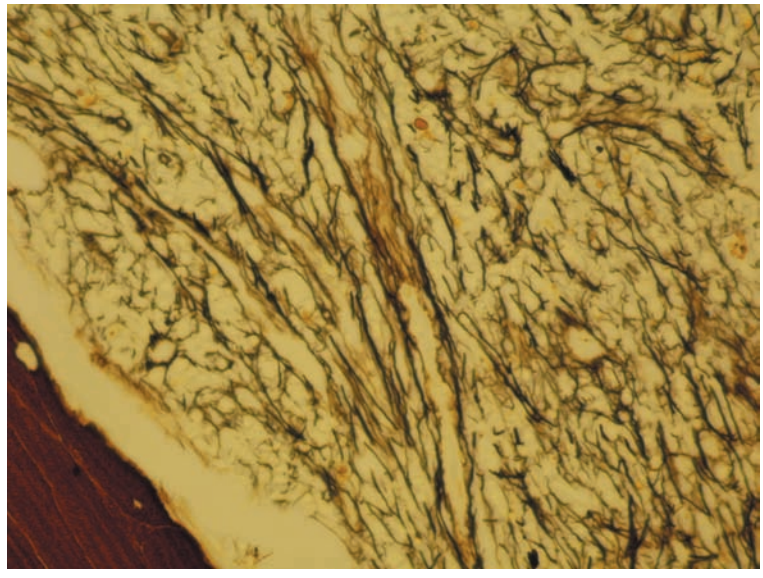
sarcoma) or predominantly of cells showing monocytic differentiation (monocytic sarcoma). Sometimes eosinophils are prominent. If the extramedullary tumour formation occurs in a patient known to have AML there is generally no diagnostic difficulty. If it occurs in advance of AML there can be confusion with non-Hodgkin lymphoma or other tumours, which is resolved by immunohistochemistry. If a patient presenting with myeloid sarcoma is found to have t(8;21) (or another of the recurrent genetic abnormalities described above) the case should be assigned to the appropriate genetic category.

Myeloid proliferation related to Down syndrome

Abnormal myeloid proliferation in Down syndrome takes two forms, transient abnormal myelopoiesis



(e)



(f)

Fig. 3.55 (*Continued*) (e) Clusters of erythroblasts are highlighted by binding to a lectin derived from *Ulex europaeus*. Immunoperoxidase ×60. (f) Heavy reticulin deposition, grade 4 of 4 since collagen was also present. Reticulin stain ×60.

(TAM) and acute megakaryoblastic leukaemia. TAM occurs in the fetus and the neonate and, although it is neoplastic in nature, spontaneous remission usually occurs. Acute megakaryoblastic leukaemia occurs in infants and, although it does not remit spontaneously, it is very sensitive to chemotherapy and has a good prognosis. Both conditions are associated with an acquired *GATA1* mutation in the neoplastic cells.

Transient abnormal myelopoiesis

Neonates with Down syndrome have been observed to have a condition that closely resembles acute leukaemia but that usually resolves spontaneously to be later followed, in some but not all cases, by acute leukaemia that does not show spontaneous resolution. This phenomenon has sometimes been regarded as a leukaemoid reaction. However, in a number of cases there has been an

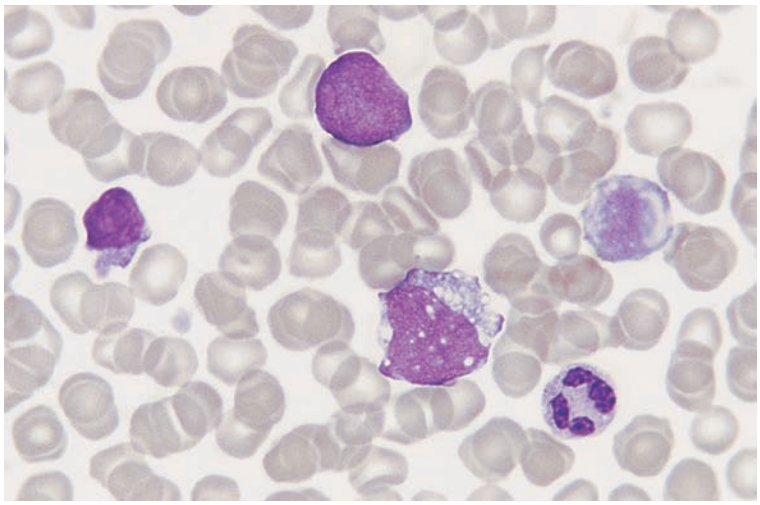


Fig. 3.56 PB of a neonate with transient abnormal myelopoiesis of Down syndrome showing a neutrophil, a giant platelet, an unidentifiable abnormal cell, a blast cell and a micromegakaryocyte. The blast cells were demonstrated to be megakaryoblasts by immunophenotyping. MGG ×100.

additional clonal cytogenetic abnormality in the proliferating cells and, by molecular analysis, clonality can be shown in all [306–309]. It is now clear that TAM is actually a spontaneously remitting leukaemia with mutation of the *GATA1* gene being consistently present [310]. Identical twins with transient abnormal myelopoiesis were found to have identical *GATA1* mutations, indicating intrauterine transfer of a cell carrying the mutation from one twin to the other [311]. *GATA1* mutations are found in about 4% of neonates with Down syndrome [312]. Babies with TAM often have hepatosplenomegaly and anaemia; less often they have liver failure or hydrops fetalis [313] with pericardial and pleural effusions and ascites. Some affected fetuses die *in utero* and some babies die as a result of bone marrow dysfunction and liver failure. A higher WBC, premature birth, abnormal liver function tests, increased conjugated bilirubin, ascites, haemorrhagic manifestations and, not surprisingly, failure to enter spontaneous remission all correlate with early death [309,314,315]. In one study 23% of babies with TAM died in the first 6 months of life [315] and in a second the death rate was 15% [314]. Transient abnormal haemopoiesis can also occur in mosaic Down syndrome and in babies without Down syndrome who have both trisomy 21 and a *GATA1* mutation confined to their haemopoietic cells [316]. Babies with mosaic Down syndrome and with trisomy 21 confined to haemopoietic cells are also at risk for subsequent development of acute megakaryoblastic leukaemia [316].

Haematological features, in addition to anaemia, may include thrombocytopenia with platelet counts sometimes as low as 10×10^9/l. In other babies there is thrombocytosis with counts above 1000×10^9/l having been reported [308]. There may be giant platelets and circulating micromegakaryocytes. Blast cells are present in the blood and marrow in variable numbers, but often they are quite numerous; they may be more numerous in the blood than in the marrow (Figs 3.56 and 3.57). The abnormal cells are often megakaryoblasts but sometimes have features of primitive erythroid cells or of basophiloblasts [317]. The count of mature basophils is occasionally greatly elevated.

The immunophenotype in TAM is characteristic [314,318]. Blasts are positive for CD7, CD13, CD33, CD34, CD36, CD38, CD71 and CD117. CD41, CD42b and CD61 are usually expressed and CD56 is expressed in about 80% of cases. There may also be expression of glycophorin A (CD235a). In one study blast cells expressed receptors for erythropoietin, thrombopoietin and interleukin 3 [314] but in another erythropoietin receptor was not detected [318].

The mutations that have been observed in *GATA1* lead to expression only of the short form of GATA1, GATA1s, rather than both the short and the full length forms. GATA1s is less active than full length

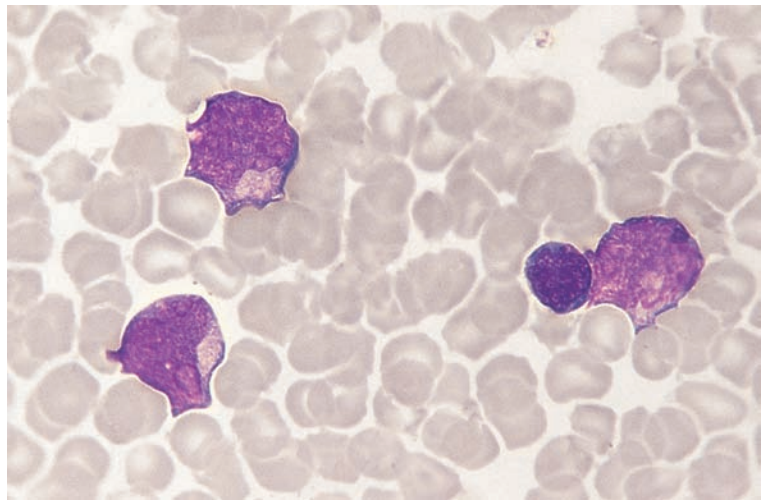


Fig. 3.57 BM film of the same patient as shown in Fig. 3.56 showing a lymphocyte and three pleomorphic blasts. MGG ×100.

GATA in promoting megakaryocyte maturation. It is likely that the leukaemogenic mutation in TAM and acute megakaryoblastic leukaemia involves interaction of a mutated *GATA* gene and one or more chromosome 21 genes that are present as three rather than two copies. One such gene that may be implicated is *ERG* at 21q22, expression of which favours megakaryocyte differentiation [319].

About a fifth of babies with TAM show an acquired clonal cytogenetic abnormality, in addition to the constitutional trisomy 21 or related abnormality. Among these abnormalities, complex rearrangements are prominent. When remission occurs, the acquired abnormality is no longer detectable. The presence of a cytogenetic abnormality is not predictive of future leukaemia or of prognostic significance [314].

A prospective study of 146 babies with TAM found an overall 5-year survival of 85% and an event-free survival of 63% [314]. In those with adverse prognostic features (high WBC, ascites, bleeding diathesis and preterm birth), low dose cytarabine therapy was demonstrated to be of benefit [314]. Otherwise, since spontaneous remission is to be expected (84%), only supportive management is required. Transformation to AML occurred in 23% and these infants had a significantly better 5-year survival (91%) than Down syndrome infants who presented with acute leukaemia without documented TAM (70%) [314].

Myeloid leukaemia associated with Down syndrome

In a fifth to a quarter of babies who have shown evidence of TAM, acute myeloid leukaemia subsequently develops [308,309,314], usually after an interval of about 2 years and sometimes with an intervening phase in which myelodysplastic features are apparent. AML also occurs in babies who have not had documented TAM. Retrospective analysis has shown *GATA1* mutations to have been present at birth in Down syndrome babies who subsequently developed AML but in whom blood film examination to exclude TAM had not been done at birth [320]. As would be expected, not all babies with a *GATA1* mutation at birth developed AML [320]. All cases of Down syndrome-related AML show a *GATA1* mutation [321] and when there has been preceding TAM the mutation is the same [310,314]. Multiple independent *GATA1* mutations may be present [320].

In one study of 24 patients, onset was at 8–38 months (median 21 months) with previously documented TAM in a third and MDS in a half [322]. In the WHO classification, MDS and AML are grouped together as 'myeloid leukaemia associated with Down syndrome' [323]. The acute leukaemia of Down syndrome is usually FAB type M7 (Fig. 3.58) but occasionally M1 or M2 [309,324]. The megakaryoblasts often show no cytological signs of differentiation but in a minority of cases there are cytoplasmic blebs and occasionally there is differentiation to dysplastic megakaryocytes [322].

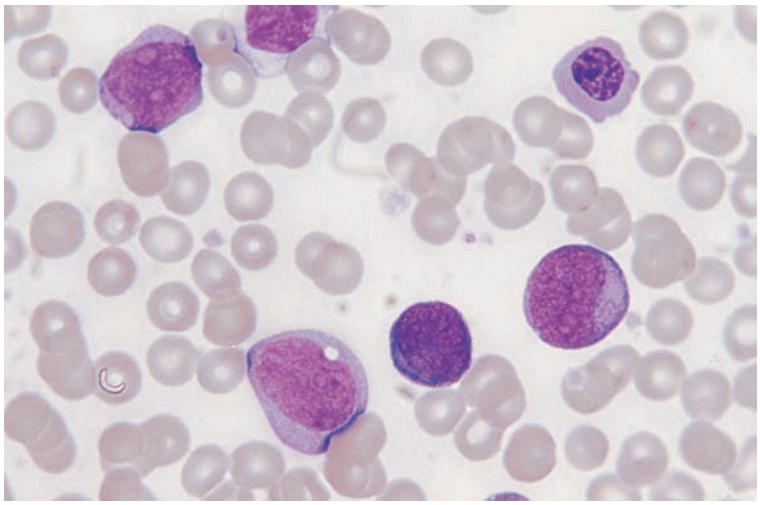


Fig. 3.58 PB film from a patient with Down syndrome with FAB M7 AML; blasts are pleomorphic with no specific distinguishing features. The nature of the leukaemia was demonstrated by showing expression of CD61. MGG ×100.

Immunophenotype shows expression of platelet antigens such as CD41, CD42 and CD61 [322,325, 326]. Myeloid antigens such as CD13 and CD33 are expressed in approaching 80% of cases and coexpression of CD7 and CD117 is even more common [318,322]. Glycophorin is expressed in about half of patients [322]. CD34 is expressed in about half (in comparison with about 90% in TAM) and CD56 may be expressed [318]. CD38 and CD71 are expressed.

Those infants who initially showed an acquired chromosomal abnormality may show the same or a different abnormality when leukaemia subsequently occurs [309]. Those initially showing only the constitutional abnormality often also show an acquired abnormality at the AML stage of the disease. In one study the presence of clonal cytogenetic abnormalities during TAM correlated with future development of leukaemia [309] but in a second larger study it did not [314]. Cytogenetic abnormalities, found in three quarters of children with Down syndrome and AML, differ from those in non-Down syndrome childhood AML [327]. The common balanced translocations are uncommon in Down syndrome and instead unbalanced abnormalities are common: dup(1q), del(6q), del(7p), dup(7q), +8, +11, del(16q) and acquired +21 [327].

Microarray analysis shows some differences between TAM and the later AML. *PRAME* is expressed only in AML and *CDKN2C*, the effector of GATA1-mediated cell cycle arrest, is increased whereas *MYCN* is expressed at a higher level in TAM than in AML [328]. Activating mutations of *JAK3*, which are uncommon in TAM, may play a role in progression to AML [329].

The prognosis of AML in Down syndrome is particularly good, at least in infants and children who develop AML under the age of 4 years, and reduced intensity treatment may therefore be given [81,330]. Blasts are very susceptible to cytarabine, anthracyclines, mitoxantrone and etoposide [324]. Complete remission rate is around 90% and 10-year survival and event-free survival is nearly 80% [322].

Blastic plasmacytoid dendritic cell neoplasm

In the 2001 WHO classification a rare, poor prognosis neoplasm with distinctive clinical and immunophenotypic features but of uncertain lineage was designated blastic natural killer lymphoma. Elsewhere it was designated CD4+CD56+ haematodermic neoplasm. It was subsequently recognized as being of plasmacytoid dendritic cell lineage [331] and in the 2008 WHO classification it has been designated blastic plasmacytoid dendritic cell neoplasm [332]. There is sometimes an associated MDS or later development of AML suggesting an origin from a myeloid stem cell.

Clinical and haematological features This is a clinically aggressive neoplasm. Patients are mainly elderly and more often male. Presentation is often with cutaneous lesions (nodules or tumours).

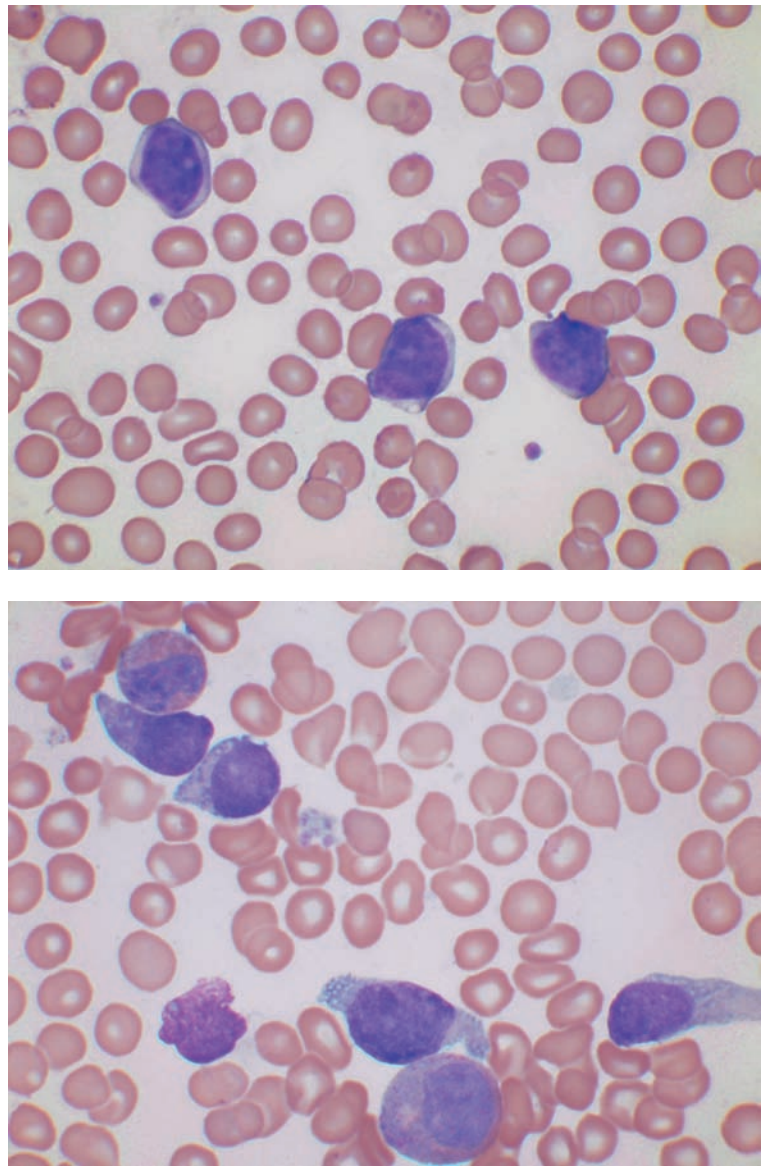


Fig. 3.59 Blastic plasmacytoid dendritic cell neoplasm; (a) PB film showing blast cells with no signs of differentiation; (b) BM film showing blast cells, some of which have cytoplasmic tails or clusters of small cytoplasmic vacuoles. This patient had bone marrow infiltration at presentation with peripheral blood dissemination being a later feature. MGG ×100.

Some patients also have splenomegaly and lymphadenopathy and, in those with isolated cutaneous lesions, there is early dissemination to bone marrow, peripheral blood, lymph nodes and extranodal sites. Central nervous system involvement may be apparent at presentation or at relapse. Except for those with isolated cutaneous lesions, the bone marrow is usually involved and blood involvement is often present (Fig. 3.59). Cytopenias are common. Cells are either small to medium or medium to large in individual patients with regular or irregular nuclei and a variable amount of vacuolated, agranular cytoplasm with an irregular surface membrane. Prominent cytoplasmic tails are often present and there may be small vacuoles.

Immunophenotype The immunophenotype is distinctive and of considerable diagnostic importance. There is almost always expression of CD56 in the absence of B-lineage and most myeloid antigens.

The only T-lineage antigens expressed are CD4 and often CD7. Expression of CD4, CD56, CD123 and CD45RA with lack of expression of CD45RO and CD116 has been found to be highly specific for this condition [333]. CD45 expression is usually low [331]. There is often also expression of CD36, CD38, CD68, CD71 and HLA-DR [331,333]. CD43 and plasmacytoid dendritic cell-associated markers, CD68, CD303, TCL1 and CLA, are expressed [332]. Some cases have shown expression of TdT [334] and some of CD33.

Cytogenetic and molecular genetic features Cytogenetic analysis shows clonal abnormalities, usually complex, in two thirds of patients [335]. Rearrangements preferentially involve 5q, 17p, 13q, 6q, 15q and chromosome 9 with del(5q) being a frequent feature [331,335].

Other genetic subtypes that have not yet been incorporated into the WHO classification of acute myeloid leukaemia

Acute myeloid leukaemia with t(8;16)(p11.2;p13.3); MYST3-CREBBP

Acute myeloid leukaemia associated with t(8;16)(p11.2;p13.3) (Fig. 3.60) is a rare variant of AML, comprising fewer than 1% of cases [14]. Most cases occur *de novo* but some are therapy related [149,291]. Therapy-related cases have followed both radiotherapy and exposure to a variety of types of chemotherapeutic agent including alkylating agents and topoisomerase II-interactive drugs [22,23]. Most patients are young and some are infants. In the WHO classification, cases are categorized either as t-AML or as AML, NOS.

Clinical and haematological features There is usually monocytic differentiation [336–338]. More than half the reported cases have been FAB type M5, particularly M5a with the majority of the remainder being FAB M4. In therapy-related cases there is a short latent period and usually no myelodysplastic phase. Many *de novo* cases have been in infants and children and if therapy-related cases are excluded the median age is low. Spontaneously remitting congenital leukaemia has occurred [231]. A prominent haemostatic abnormality, sometimes interpreted as disseminated intravascular coagulation and sometimes as increased fibrinolysis, is usual. Extramedullary disease, including skin infiltration, is common [338]. The prognosis appears to be relatively poor.

The majority of cases have shown haemophagocytosis by leukaemic cells (Fig. 3.61), particularly erythrophagocytosis, to the extent that the first case described was designated malignant histiocytosis. In other cases this was only a minor feature. Monoblasts are often granulated but Auer rods are not a feature.

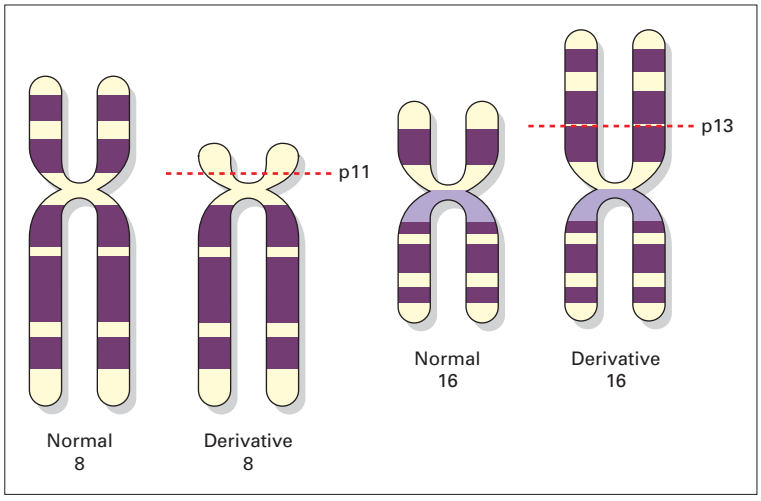


Fig. 3.60 Diagrammatic representation of t(8;16)(p11;p13). (Modified from reference 2.)

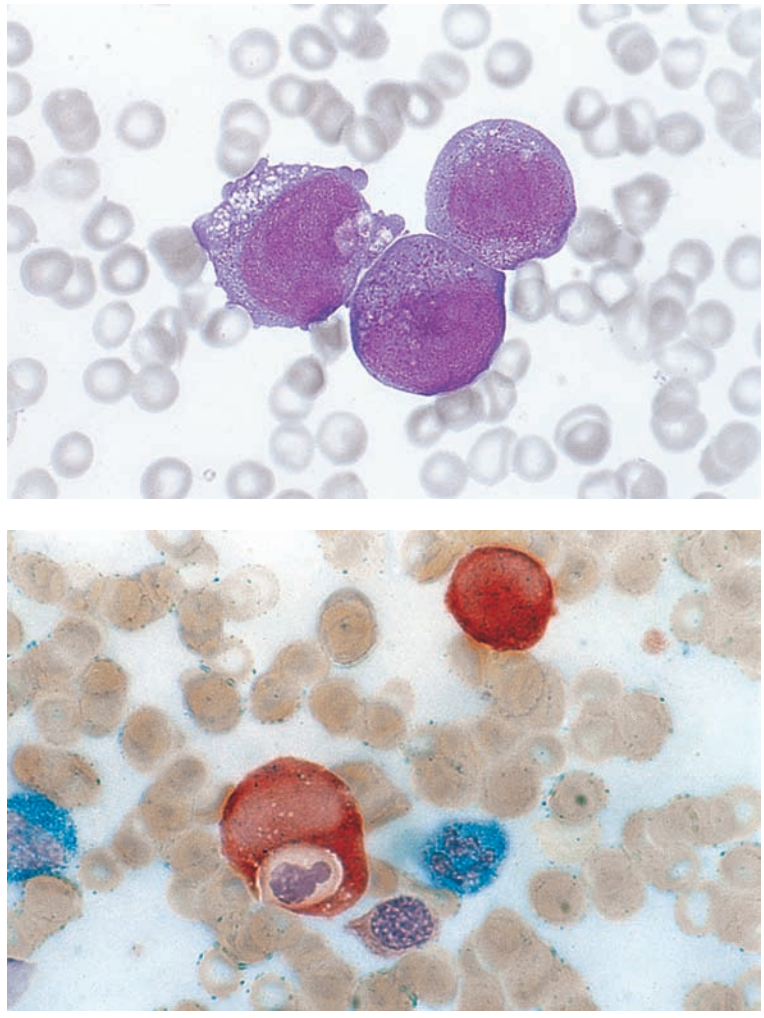


Fig. 3.61 BM aspirate from a patient with FAB M5 AML associated with t(8;16)(p11;p13). (a) Three monoblasts. MGG ×100. (b) A neutrophil and two monoblasts, one of which has phagocytosed a neutrophil. Double esterase reaction: CAE, blue; α-naphthyl acetate esterase, brown ×100. (By courtesy of Professor Daniel Catovsky, London.)

Immunophenotype The characteristic immunophenotype is positivity for HLA-DR, CD33 and CD15 [337,338]. CD13 and CD14 are positive in about half of reported cases. CD34 and TdT are usually negative. As in other cases of FAB M4 and M5 AML, there may be expression of the natural killer marker, CD56.

Cytogenetic and molecular genetic features This translocation involves *CREBBP*. There is fusion of the *MYST3* (previously known as *MOZ*) gene on chromosome 8 with the *CREBBP* gene (previously known as *CBP*) from 16p13 [339]. It appears to be the *MYST3-CREBBP* fusion gene that is important in leukaemogenesis rather than the *CREBBP-MYST3* gene [340]. Secondary chromosomal abnormalities include trisomy 1 and trisomy 8 [210].

Acute myeloid leukaemia with the same clinical and cytological features has also been associated with variant translocations involving chromosome 8, including t(6;8)(q27;p11), t(8;14)(p11;q11.1), t(8;19)(p11;q13), t(8;22)(p11;q13) and t(3;8;17)(q27;p11;q12) [338], and also with an inversion of chromosome 8, inv(8)(p11q13) [341]. One case of t-AML associated with t(8;22)(p11;q13) has been reported [342]. Haemophagocytosis by leukaemic blast cells has also been a feature of cases with t(8;22) and inv(8) [341,343]. *FLT3*-ITD has been

described in one patient with inv(8)(p11q13) and *MYST3-NCOA2* (previously known as *MOZ-TIF2*) [344].

Acute myeloid leukaemia with t(16;21)(p11;q22); FUS-ERG

t(16;21)(p11;q22) is mainly associated with FAB M1 and M2 AML. In the WHO classification, cases are categorized as AML, NOS. Haemophagocytosis by leukaemic blasts is a common feature [345,346]. The most common secondary cytogenetic abnormality is trisomy 10. The mechanism of leukaemogenesis is formation of a *FUS-ERG* fusion gene. Despite a high rate of complete remission, prognosis is poor with the median survival being about a year [47]. The *FUS-ERG* fusion gene can be detected by RT-PCR.

Acute myeloid leukaemia with t(9;22)(q34;q11.2); BCR-ABL1

Patients with AML and t(9;22)(q34;q11.2) and *BCR-ABL1* comprise fewer than 1% of cases of AML but are over-represented in the FAB M0 category where they may represent more than a quarter of cases [347]. Most cases occur *de novo* but therapy-related cases are recognized [291,348]. In the WHO classification cases are categorized as AML, NOS.

With chemotherapy alone, prognosis has been poor with only 30–40% of patients achieving a complete remission and median survival of less than a year [47,349]. Five-year survival was negligible in one series of patients [349] but a group of 47 MRC trial patients had a 10-year survival of 16% [29]. Even imatinib-treated patients have a poor prognosis due to failure to achieve a complete remission and short remission duration [349]. The prognosis of therapy-related cases is, if anything, even worse [291].

BCR-ABL1-positive AML should be distinguished from blast crisis of a previously undiagnosed CGL. Patients with AML are less likely to have splenomegaly although massive splenomegaly can occur [349]. The basophil count is much less often elevated [349]. In cases of AML there is reversion to a normal karyotype after effective chemotherapy whereas in blast crisis of CML there is reversion to Ph positivity.

Rarely Ph-positive AML develops in a patient with MDS with the t(9;22) being a second event [350].

Clinical and haematological features Clinical features differ from those of Ph-negative AML in that hepatomegaly and splenomegaly are more common [351]; nevertheless, only a minority of patients have splenomegaly [349]. The basophil count can be increased but in the majority of patients it is not [349]. Cases are usually FAB M0 or M1 and less often M2, M4, M6 or M7. MPO activity may be weak or absent [137,352]. Auer rods are uncommon. Only a minority of patients have an increase of bone marrow basophils [349].

Immunophenotype There are no specific immunophenotypic features. There is usually expression of CD13, CD33 and CD34 but not TdT [349]. Lymphoid antigens such as CD19 are sometimes expressed.

Cytogenetic and molecular genetic features The t(9;22)(q34;q11.2) rearrangement (Fig. 3.62) is the same as that seen in CGL (see page 266). Variant translocations also occur, e.g. t(9;11;22)(q34;q12;q11.2) and t(9;22;21)(q34;q11;p11) [349]. The majority of patients have secondary clonal abnormalities or clonal evolution [349]. The commonest associated chromosomal aberrations are monosomy 7, trisomy 8 and 19 and duplication of the Ph chromosome, der(22)t(9;22) [210]. Other secondary abnormalities recognized in blast crisis of CGL can also occur, e.g. i(17)(q10) and inv(3)(q21q26.2) [349]. However, the prevalence of typical blast crisis secondary abnormalities is lower than in blast crisis, suggesting that molecular mechanisms in *de novo* Ph-positive AML differ [349].

A number of FISH strategies are available for detection of t(9;22) (see page 266 and Fig. 3.68).

The molecular mechanism is formation of a *BCR-ABL1* fusion gene. The *BCR* breakpoint occurs more frequently in the major breakpoint cluster region (M-BCR), the same breakpoint as in CGL, rather than in the minor breakpoint cluster region (m-BCR), characteristic of ALL.

Acute myeloid leukaemia arising in a germ cell tumour with i(12p)

A unique and rare type of AML is that which derives from a germ cell tumour, usually a mediastinal germ cell tumour with yolk sac elements occurring in males [178,353–355,] but occasionally arising in

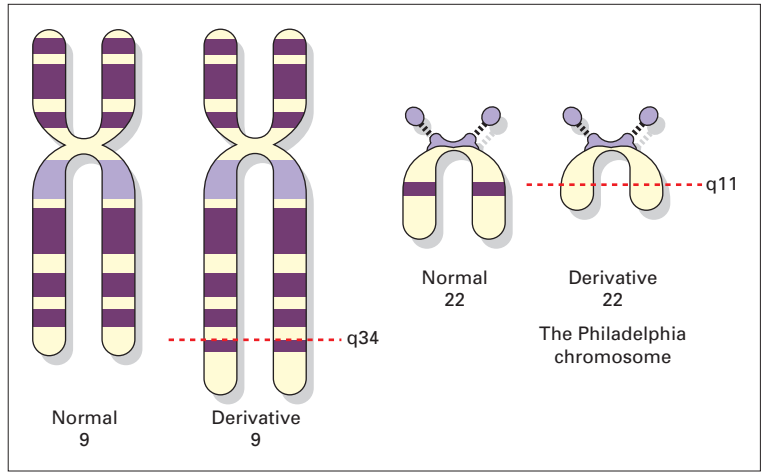


Fig. 3.62 Diagrammatic representation of t(9;22)(q34;q11.2). (Modified from reference 2.)

an ovarian germ cell tumour [356]. An isochromosome of 12p is specifically associated with germ cell tumours and is also found in the leukaemic cells indicating a common clonal origin. The molecular mechanism of leukaemogenesis has not yet been defined; however, the critical region appears to be 12p11.2–12.1. The commonest associated chromosomal abnormalities are +X (either acquired or as a constitutional abnormality in Klinefelter syndrome) and trisomy 8 [357]. A variety of FAB types have been described. The commonest is M7 but M4, M5, M6, malignant histiocytosis and MDS (FAB refractory anaemia with excesss of blasts (RAEB) category) have also been observed. AML and the germ cell tumour may present simultaneously or AML may appear after apparently successful treatment of the germ cell tumour. There may also be an association between MDS and mediastinal germ cell tumour; refractory anaemia with ring sideroblasts [358] and RAEB [359], both associated with i(12p), have been described following successful treatment of the primary tumour. Differentiation may be lymphoid as well as myeloid, precursor-B acute lymphoblastic leukaemia with i(12p) also having been described following a mediastinal germ cell tumour [360].

Other acute myeloid leukaemia with other recurrent cytogenetic abnormality

There are a large number of recurrent cytogenetic/genetic abnormalities that appear to define specific entities, each of which constitutes well below 1% of cases of AML. Unless they are therapy related, they fall into the WHO category of AML, NOS. They are summarized in Table 3.7.

Other recurring genetic abnormalities that should be noted in cases of acute myeloid leukaemia

Further recurrent mutations have been described in AML, both in the molecularly defined entities incorporated into the WHO classification and in other cases, including cases with normal cytogenetic analysis. In the case of *NPM1* and *CEBPA* mutations, the mutations have been recognized as defining provisional WHO entities. In other instances a molecular genetic abnormality might be a defining abnormality of a specific entity but the evidence is not yet available. Some recurring mutations cannot easily be incorporated into a classification of AML since they are not mutually exclusive and in some instances represent a secondary rather than primary abnormality [269]. *FLT3*-ITD and *FLT3*-TKD mutations are examples of secondary abnormalities, occurring in multiple subtypes of AML and not necessarily being present at relapse. The prevalence of various mutations shows both positive and negative correlation with the presence of other mutations so that large series of patients and multivariate analysis are necessary to establish the true prognostic significance of each [384].

Table 3.7 Some of the genetic categories of acute myeloid leukaemia not involving 11q23 or the *MLL* gene [24,89,203,232,239,243,253–257,361–383].

Translocation	Type of leukaemia	Molecular event	Reference
t(1;3)(p36;q21)	t-AML and t-MDS	A shortened form of *PRDM16* (*MEL1*) is expressed and inhibits myeloid differentiation	[362]
t(1;11)(q23;p15)	FAB M2 (t-AML)	*NUP98-PRRX1* (*PMX1*)	[363]
t(1;12)(q21;p13)	A case of FAB M2 AML	*ETV6* (*TEL*)-*ARNT*	[364]
t(1;21)(p36;q22)	t-AML	*RUNX1* rearranged	[365]
t(2;11)(q31;p15)		*HOXD13-NUP98*	
t(3;5)(q25.1;q34)	AML or MDS	*NPM1-MLF1*	[253]
t(3;12)(q26;p13)	MDS, AML, blast crisis of CGL	Heterogeneous at 3q26, mainly *ETV6* (*TEL*) at 12p13; *MDS1/EVI1-ETV6*	[255,361]
t(3;21)(q26;q22)	AML, either t-AML or following MPN; blast crisis of CGL	Heterogeneous (mainly *RUNX1-RPL22* (*EAP*), *RUNX1-EVI1* and *RUNX1-MDS1*)	[243,256,257]
t(4;12)(q11-22; p13)	FAB M0 and M2 AML	*CHIC2* (*BTL*)-*ETV6*	[366]
Cryptic t(5;11)(q35;p15.5), often with 5q–	Childhood AML(FAB M1, M2 or M4)	*NSD1-NUP98* and *NUP98-NSD1*	[367]
t(5;12)(q31;p13)	Case of AML with eosinophilia	*ACSL6* (*ACS2*)-*ETV6*	[368]
t(7;11)(p15;p15)	FAB M2 AML, in Chinese and Japanese; trilineage myelodysplasia [239]	*NUP98-HOXA9* or *NUP98-HOXA13*	[369,370]
t(7;12)(p15;p13)		*ETV6* rearranged	
t(7;12)(q36;p13), often cryptic	*c.* 30% of infant AML (with poor outcome)	*HLXB9-ETV6* or *HLXB9* overexpression	[371]
t(8;11)(p11.2;p15)	FAB M1 AML(one case)	*NUP98-WHSC1L1* (*NSD3*)	[372]
t(8;22)(p11;q13)	FAB M5 AML	*MYST3* (*MOZ*)-*EP300* and *EP300-MOZ*	[373]
t(9;11)(p22;p15)	AML	*PSIP1* (*LEDGF*)-*NUP98*	[374]
t(9;12)(q34;p1?) (cryptic)	AML with trilineage dysplasia and clonal eosinophils	*ETV6-ABL1*	[375,376]
t(10;11)(p12-13; q14-21)	FAB M0, M1, M2, M4 and M5 AML MPAL	*PICALM-MLLT10* (*AF10*), less often *MLLT10* (*AF10*)-*PICALM*	[223,232,377]
t(10;16)(q22;p13)	Childhood FAB M5a AML	*MYST4-CREBBP* (*MORF-CBP*)	[378]
inv(11)(p15q11)		*NUP98-DDX10*	
t(11;20)(p15;q11)		*NUP98-TOP1*	
t/dic(12;13)(p11.2-13; p11-q14)	Various FAB categories (M1, M1, M2, M5) and MDS	*ETV6-CDX2*	[361,379]
t(12;15)(p13;q25)	A case of FAB M2 AML	*ETV6-NTRK3* (*TRKC*)	[380]
t/dic(12;20)(p12-13; p11.2-q13)†	AML or MDS	Not known	[361]
t(12;22)(p13;q11)	Various FAB categories (M1, M4, M7)	*MN1-ETV6*	[381]
t(16;21)(p11;q22)	AML (FAB M1, M2, M4, M5, M7) and MDS	*FUS* (*TLS*)-*ERG*	[382]
t(16;21)(q24;q22)	t-AML, FAB M2 or M2Eo; t-MDS; *de novo* MDS; or AML	*RUNX1-CBFA2T3(MTG16)*	[24,89]
t(X;6)(p11;q23)	FAB M0Baso in infants	Not known	[383]
Normal	A case of FAB M0 AML	*SET-NUP214* (*CAN*)	

AML, acute myeloid leukaemia; CGL, chronic granulocytic leukaemia; FAB, French–American–British (classification); MDS, myelodysplastic syndrome; MPAL, mixed phenotype acute leukaemia; MPN, myeloproliferative neoplasm; t-AML, therapy-related AML; t-MDS, therapy-related MDS.

Both *de novo* and therapy-related AML can be associated with *MLL*-PTD, with or without trisomy 11. In this rearrangement, exons 5–11 or 5–12 are inserted into intron 4 and the partially duplicated gene is transcribed; the wild-type gene is not expressed and this contributes to or causes the leukaemic phenotype [385]. Prevalence rises with age and has varied from 3% to 6% in a large series of patients [386]. In patients with normal cytogenetics it is higher, ranging from 5% to 8% in large series [386]. The highest prevalence is in patients with trisomy 11, among whom about a third have *MLL*-PTD [386]. This genetic abnormality has been associated with a poor prognosis [194,386,387] but in a group of patients with *MLL*-PTD and normal cytogenetics who received intensive treatment the prognosis was not adverse [388]. *FLT3*-ITD may coexist [386].

It is important that, when facilities are available, the possibility of gene mutations is investigated and noted since some of the mutations are of prognostic importance, and thus may influence choice of treatment, and some may provide a molecular target for treatment. *FLT3*-ITD is an example of an abnormality of adverse prognostic significance that also offers the possibility of targeted therapy. Some of these recurring genetic abnormalities are summarized in Table 3.8.

Table 3.8 Recurring genetic abnormalities that influence outcome in acute myeloid leukaemia [6,75,157,194,269,384,386–392].

Gene that is mutated or abnormally expressed	Associations	Frequency	Significance
KIT mutation	t(8;21) and inv(16)/t(16;16)		Adverse in patients with t(8;21), inv(16) and t(16;16)
FLT3-ITD (internal tandem duplication)	Many categories; common in cytogenetically normal, acute promyelocytic leukaemia (particularly the variant form) and with t(6;9); found in a third of patients with trisomy 11 [389]	20–40%; 36% in those with a normal karyotype [384]	Adverse in multiple genetic subtypes including acute promyelocytic leukaemia and mutated *NPM1*; adverse with normal cytogenetics [390]
FLT3-TKD (tyrosine kinase domain mutation)		9% in those with a normal karyotype [384]	Data are conflicting [75,157,391]
MLL-PTD (partial tandem duplication) [386]	Found in a third of patients with trisomy 11 and in 5–10% of cases with normal karyotype	3–6%; 5–8% in those with a normal karyotype	May be adverse
WT1 mutation			Adverse in association with normal karyotype
NRAS, *KRAS*		Common	Uncertain
JAK2 V617F mutation [392]	Associated particularly with FAB type M2	4% of patients with a normal karyotype	
BAALC overexpression (defined as above the median value) [384,390]	Associated with *FLT3*-ITD, *CEBPA* mutation and high *ERG* expression		Adverse in those with normal karyotype, independent of *FLT3*-ITD and *NPM1* and *CEBPA* mutations
ERG overexpression [390]		37% in those with a normal karyotype [384]	Adverse in those with normal karyotype
MN1 expressed [390]			Adverse in those with normal karyotype

FAB, French–American–British (classification).

Acute leukaemias of ambiguous lineage

This WHO category includes both cases that do not express any clear lineage-associated markers (designated acute undifferentiated leukaemia) and those that show significant expression of markers of more than one lineage (mixed phenotype acute leukaemia, MPAL) [393]. The latter category includes cases that were previously designated either biphenotypic or bilineal acute leukaemia. The criteria for categorization as MPAL have been altered in the light of new information on the lack of lineage specificity of some immunophenotypic markers. Criteria used to assign a case to the MPAL category may be derived from flow cytometry immunophenotyping, immunohistochemistry or cytochemistry. They are summarized in Table 3.9 together with the criteria for a diagnosis of acute undifferentiated leukaemia. About a third of MPAL cases are Ph positive [394,395]. The second commonest group is associated with a variety of abnormalities with an 11q23 breakpoint, particularly t(4;11) and del(11)(q23). These two groups are assigned to specific categories. Mixed phenotype acute leukaemias generally appear to have a poor prognosis, possibly but probably not only, because of the frequent association with an adverse karyotype.

Table 3.9 WHO 2008 criteria for a diagnosis of mixed phenotype acute leukaemia [393].

Lineage	Criteria
Myeloid	Myeloperoxidase (any technique) *or* Expression of at least two of: diffuse non-specific esterase activity, CD11c, CD14, CD64 or lysozyme
T	Cytoplasmic CD3 (but not expression only of CD3ζ) or surface membrane CD3
B	Strong CD19 with at least one of: CD79a*, cCD22 or CD10* *or* Weak CD19 with strong expression of at least two of: CD79a*, cCD22 or CD10* *or (rarely)* Strong evidence of B lineage despite CD19 negativity
Undifferentiated	No expression of cCD3, myeloperoxidase, cCD22 or cCD79a, absence of strong expression of CD19

c, cytoplasmic.
* But in assigning B-lineage to T-lineage cells, CD79a and CD10 should not be considered since both can also be expressed by T lymphoblasts.

Acute undifferentiated leukaemia

Little is known about this rare category of acute leukaemia. The diagnosis should only be made after extensive investigation to exclude a more specific diagnosis. There may be expression of CD34, TdT, CD38 and HLA-DR but not of the lineage-specific markers listed in Table 3.9.

Mixed phenotype acute leukaemias

Mixed phenotype acute leukaemia with t(9;22)(q34;q11.2) and BCR-ABL1

t(9;22)(q34;q11.2) is associated with a variety of types of biphenotypic leukaemia, particularly with cases showing evidence of myeloid and B-lymphoid differentiation but also with occasional cases with B- and T-lymphoid or myeloid, B-, and T-lymphoid differentiation [395]. The majority of cases are adult although childhood cases do occur. Morphologically, cells more often appear lymphoid but in some cases they appear myeloid and sometimes blast cells are dimorphic. Presentation is with cells of the neoplastic clone showing simultaneous expression of lymphoid and myeloid antigens. Diagnosis requires that: (i) criteria for MPAL are met; (ii) either t(9;22) or *BCR-ABL1* is demonstrated; and (iii) the patient is not known to have had chronic granulocytic leukaemia. There are often additional cytogenetic abnormalities, sometimes with a complex karyotype.

Mixed phenotype acute leukaemia with t(4;11)(q21;q23) and MLL-MLLT2 or other rearrangement of 11q23/MLL

t(4;11)(q21;q23) is associated with ALL with an early B precursor phenotype, with acute monoblastic leukaemia and with MPAL. In *MLL*-associated

MPAL the myeloid component is often of the monocytic lineage. Although the ALL component is usually early B precursor (often with coexpression of CD15) it is occasionally common or pre-B ALL. Some patients have either biphenotypic blasts or two blast populations at diagnosis, while others present with ALL and either have blasts of myeloid phenotype emerging early during the course of treatment or subsequently relapse with a myeloid phenotype. In addition to t(4;11), other translocations with an 11q23/*MLL* breakpoint have also been associated with MPAL. Other partner chromosomes have included 9, 17 and 19 [200,395,396].

A significant proportion of the rare leukaemias associated with t(10;11)(p13;q21), involving the *MLLT10* and *PICALM* genes, are of mixed phenotype, particularly myeloid and T lineage but occasionally myeloid, T and B lineage [397].

Mixed phenotype acute leukaemia, B/myeloid, not otherwise specified

This rare type of leukaemia has no specific distinguishing characteristics. Chromosomal abnormalities that have been described include del(6p), abnormalities of 12p11.2, del(5q), monosomy 7, structural abnormalities of chromosome 7 and aneuploidy including near tetraploidy [393,398].

Mixed phenotype acute leukaemia, T/myeloid, not otherwise specified

This rare type of leukaemia has no specific distinguishing characteristics. Complex chromosomal rearrangements may be observed.

Mixed phenotype acute leukaemia, not otherwise specified, rare types

This category includes the rare examples of B/T-lineage MPAL and B/T/myeloid MPAL.

Other ambiguous lineage leukaemias

Some cases of acute leukaemia do not meet the criteria for any of the categories defined above and are best regarded as unclassifiable. Cases previously considered to represent NK cell acute leukaemia are sometimes actually blastic plasmacytoid dendritic cell neoplasm. Cases considered NK/myeloid on the basis of CD56 expression may be best considered as AML. Precursor NK cell lymphoblastic leukaemia/lymphoma is currently assigned to this WHO group.

The WHO classification of acute lymphoblastic leukaemia/lymphoblastic lymphoma

The diagnosis and classification of acute lymphoblastic leukaemia/lymphoblastic lymphoma requires: (i) recognition of a neoplastic process with cells having blastic morphology and a precursor cell immunophenotype; (ii) assignment to T or B lineage; and (iii) further categorization that recognizes real entities that differ in their molecular mechanisms, clinical and haematological features, prognosis and optimal management. Immunophenotyping contributes particularly to the first two of these three steps while cytogenetic and molecular genetic analysis provides the basis for a clinically relevant subclassification of B-lineage and, potentially, T-lineage disease. Morphology, immunophenotyping and genetic analysis are thus all of critical importance. With techniques now available 70–90% of cases of ALL have a demonstrable cytogenetic abnormality. Some cytogenetic abnormalities (such as 6q– and 9p–) are associated with both B- and T-lineage ALL, while others are confined to one lineage or are associated with a specific immunophenotype within a lineage and with other disease characteristics. Translocations with breakpoints involving immunoglobulin genes (heavy chain, κ or λ) are generally B lineage and those involving *TCR* genes are largely confined to T-lineage ALL. Hyperdiploidy is commonly associated with B-lineage ALL and is rare in T-lineage ALL.

In the 2008 WHO classification a number of cytogenetic categories of B lymphoblastic leukaemia/lymphoma have been recognized. For some of them the molecular mechanism of leukaemogenesis is not yet known. No similar categorization of T-lineage disease has yet been made. Lymphoblastic lymphomas share many features with ALL but differ in that the initial presentation is at an extramedullary site rather than in the bone marrow and peripheral blood.

It should be noted that in the 2008 WHO classification the abbreviation 'B-ALL' is used to indicate B lymphoblastic leukaemia/lymphoma. It is important that this usage is not confused with an earlier use of 'B-ALL' to refer to 'mature B-ALL', usually representing the leukaemic phase of Burkitt lymphoma.

B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities

B lymphoblastic leukaemia/lymphoma with high hyperdiploidy

This is the commonest form of childhood ALL in developed countries, being partly responsible for the typical peak in incidence of ALL in early childhood. In childhood cases, a leukaemic or preleukaemic clone may arise during intrauterine life [399]; this may occur in the majority of cases since it was observed in 10 of 11 patients in one study [400]. The WHO classification uses the term 'hyperdiploidy' but 'high hyperdiploidy' seems preferable since cases with 47–50 chromosomes are excluded [401].

Clinical and haematological features This category accounts for about a quarter of childhood ALL [402,403] but only about 7–8% of ALL in adults [404,405]. In developing countries, the incidence is lower than in developed countries. The prevalence, and probably the incidence, appears to be higher in children in Nordic countries than elsewhere with two studies having shown 33% and 46% of cases to have high hyperdiploidy (defined as a clone with at least 52 chromosomes) [406,407]. The peak incidence is between 5 and 10 years. There is a female preponderance. The WBC is relatively low. Cytological features are typically those of FAB L1 ALL. PAS-block positivity is usual. The prognosis is generally good. Children have a 10-year survival close to 90% [408]. Even in adults, who have a worse prognosis than children with high hyperdiploidy, there is a 50% 5-year survival [405]. High hyperdiploidy in adults is associated with a younger age, a lower WBC and a better prognosis than other Ph-negative categories [409]. Patients can be stratified for prognosis on the basis of the presence or absence of favourable features: age 1–10 years; female gender; and the presence of prognostically favourable trisomies, 4 and 18 [410]. The presence of unfavourable cytogenetic rearrangements, such as t(9;22), negates the otherwise good prognosis of high hyperdiploidy [411] and such cases should not be included in this category.

The leukaemic lymphoblasts are particularly sensitive to methotrexate [412].

Immunophenotype The immunophenotype is that of common ALL, i.e. CD10 is expressed whereas cytoplasmic μ chain and surface membrane immunoglobulin are not. CD34 is often expressed whereas CD45 is weak or not expressed [413]. CD66c, which is also expressed by Ph-positive ALL, is expressed [196].

Genetic and molecular genetic features The term 'high hyperdiploidy' indicates that leukaemic cells have more than 50 (but fewer than 66) chromosomes. Cases of ALL with 'low hyperdiploidy' (47–50 chromosomes) have somewhat different characteristics, including a worse prognosis [403]. Near triploidy has a good prognosis, similar to that of high hyperdiploidy to which it has been considered closely related [403]. The molecular mechanism of leukaemogenesis in high hyperdiploidy is unknown. The gain of chromosomes is not random, and is related to modal number [410,414]. In one study [414] an extra copy of 21 and X was fairly consistently present. With a modal number of 52–54, the frequency of extra chromosomes was in the order 14, 6, 4 and 18 and 17, 10. At a modal number of 56–60, frequency of further supernumerary chromosomes was in the order 8, 5, 12 and 11. The remaining chromosomes are consistently trisomic only at modal numbers greater than 68. In another study of 700 children, chromosomes most often gained were 4, 5, 10, 14, 17, 18, 21 and X, in that order [410]. The karyotypic abnormality can be demonstrated by conventional cytogenetic analysis (Fig. 3.63), by comparative genomic hybridization, by flow cytometry to quantitate nuclear DNA (the DNA index) and by multicolour FISH for combinations of the more frequent supernumerary chromosomes (X, 4, 6, 8, 10, 14, 16, 18, 20, 21) [415]. Some prognostic differences have been found, related to the specific chromosomes gained [411]. In one study gain of chromosomes 4, 6, 10 and 17 was related to a better prognosis and gain of 5 or the presence of an i(17)(q10) to a worse prognosis [411]. However, in another very large series of patients, trisomy 18, rather than triple trisomy for 4, 10 and 17, was most strongly linked to good prognosis [410]. Because of the good prognosis, screening of all patients with failed or normal cytogenetic analysis for high hyperdiploidy is advised [411]. An alternative

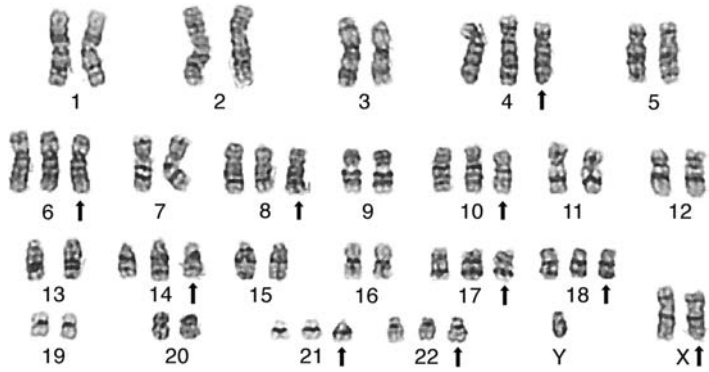


Fig. 3.63 Karyogram of a child with acute lymphoblastic leukaemia (ALL) and a high hyperdiploid clone, 56,XXY,+4,+6,+8,+10,+14,+17,+18, +21,+22; additional chromosomes are arrowed. (By courtesy of Professor Lorna Secker-Walker and the LRF UKCCG karyotype database.)

approach is to screen specifically for trisomies of 4, 10 and 17 [416,417], or for trisomies of 4 and 18 [410], since a good prognosis has been associated with these specific trisomies in different studies.

FLT3 is overexpressed [412].

B lymphoblastic leukaemia/lymphoma with t(12;21)(p13;q22); ETV6-RUNX1

This is one of the commonest subtypes of childhood ALL but was largely unrecognized until the late 1990s; the translocation is usually cryptic, since the involved portions of the two chromosomes are both small and have similar banding patterns.

Clinical and haematological features When molecular techniques are used, t(12;21)(p13;q22), previously identified as t(12;21)(p12;q22), is found in 10–30% of cases of childhood B-lineage ALL [418–423] but in only 2–4% of adult cases [411,421,424]. Affected children are aged mainly between 2 and 9 years [425] and adults are usually but not always young adults [411]. When this type of leukaemia occurs in infants and children the translocation has often occurred in intrauterine life [399,426]. It is likely that a second post-natal event is required both because of the long latent period that is observed and because the prevalence of the translocation at birth is about 100-fold the prevalence of this type of leukaemia during childhood. Thus formation of the *ETV6-RUNX1* fusion gene (previously known as *TEL-AML1*) may be an initiating event but not sufficient for transformation. Most cases have FAB L1 cytological features. The remission rate is high. Long-term survival was initially reported to be good [422,425] but in more recent studies has not differed from that of ALL in general [427–429]. Late relapses can occur, with nearly half occurring after 5 years and with relapses up to 11 years [408]. Molecular evidence (analysis of *TCR* and *IGH* genes) suggests that some apparent relapses may represent a second transforming event in an *ETV6-RUNX1*-positive stem cell or progenitor cell, rather than a true relapse [430]. This may be the explanation for the observation that, unusually for ALL, there is no plateau in the event-free survival curve [408]. Recognition of this category of ALL may have therapeutic implications since the leukaemic cells appear to be particularly sensitive to asparaginase and results may be better with regimens containing high doses of this agent [431]. Blast cells also show *in vitro* sensitivity to doxorubicin and etoposide [432].

Immunophenotype The immunophenotype may be early precursor-B, common or pre-B ALL. The relative frequencies of each vary considerably between different reported series but the common ALL phenotype is most frequent. CD34 is usually positive but expression may be weak and heterogeneous. CD20 is usually not expressed. In comparison with other precursor-B ALL, there is higher expression of CD10, CD40 and HLA-DR and lower expression of CD9, CD20 and CD86 [433]. There is lack of expression of CD66c [196]. Myeloid antigens such as CD13 and CD33 are coexpressed in at least a quarter to a half of cases [422,434,435] and, conversely, t(12;21) is found in two thirds of patients in whom these myeloid antigens are expressed [434].

Cytogenetic and molecular genetic features The translocation is difficult to detect by conventional cytogenetic analysis and may be interpreted as del(12p12) or add(12p). The above percentages relate to detection by *in situ* hybridization. Multicolour FISH can be used to elucidate complex rearrangements that can lead to the same fusion gene (see Fig. 2.13). A visible abnormality of chromosome 12 is present in approximately half of patients [423,436]. The molecular mechanism of leukaemogenesis is fusion of two transcription factor genes, *ETV6* of the *ETS* family and *RUNX1*, to form a fusion gene, *ETV6-RUNX1*, on the derivative chromosome 21. The fusion gene *RUNX1-ETV6* on der(12) is less consistently transcribed and is thus less likely to be relevant to leukaemogenesis. The other allele of *ETV6* is deleted in about three quarters of cases [425,437], this being a secondary abnormality since it may be present in only a proportion of the clonal cells. In patients without deletion of the second allele there is nevertheless failure to express wild-type *ETV6*, suggesting that loss of expression of *ETV6* is likely to be critical in leukaemogenesis [437]. In a significant proportion of cases, 13% in one series of patients [436], leukaemic cells have a second copy of the *ETV6-RUNX1* fusion gene as a result of either +der(21)t(12;21) or ider(21)(q10)t(12;21) [406]. Girls may lose one copy of chromosome X as a secondary cytogenetic abnormality [438]. Other cytogenetic abnormalities associated with a cryptic t(12;21) include del(6q), +8, abnormal 9p, del(11q), i(21q), +21 or +der(21), and translocations between chromosome 12 and a variety of partner chromosomes [435,436,439]. It is common for there to be multiple subclones at diagnosis, e.g. with deletion of *ETV6* or part of *RUNX1*, with duplication of *ETV6-RUNX1* or with trisomy or tetrasomy 21 [440]; such multiple subclones are seen in about a quarter of patients. Secondary chromosomal abnormalities are not of any prognostic significance [436].

The genetic defect can be detected by RT-PCR and by FISH using a probe for the *ETV6* gene and a chromosome 21 paint (metaphase FISH) or probes for *ETV6* and *RUNX1* (metaphase or interphase FISH) (Fig. 3.64) or two probes that together span *ETV6* (dual-colour, break-apart FISH technique). If *ETV6* and *RUNX1* probes or a split signal FISH technique are used it is possible to detect not only the translocation but also the deletion of a normal *ETV6* allele (Fig. 3.65). The case detection rate is higher with RT-PCR [406]. The detection rate by RT-PCR is somewhat higher when there is a detectable abnormality of chromosome 12 or chromosome 21 than when there is not: 56% compared to 31% [427]. The amount of MRD at the end of induction therapy, as evaluated by a limiting dilution PCR assay, is of prognostic significance [428].

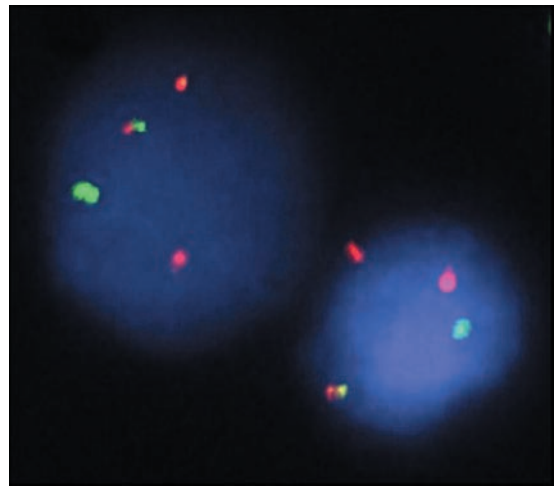


Fig. 3.64 *In situ* hybridization demonstrating *ETV6-RUNX1* fusion in interphase cells of a patient with ALL. A dual-colour translocation probe has been used. The *ETV6* probe (green) binds 5′ to the 12p13 breakpoint. The *RUNX1* probe (orange) spans the entire gene including the 12q22 breakpoint. Normal cells will thus have separate orange and green signals (two of each). The leukaemic cells shown have one normal green *ETV6* signal, one large orange signal (normal *RUNX1*), one smaller orange signal (residual *RUNX1*) and one fused double-colour *ETV6-RUNX1* signal. (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology.)

B lymphoblastic leukaemia/lymphoma with t(9;22)(q34;q11.2); BCR-ABL1

Acute lymphoblastic leukaemia associated with t(9;22)(q34;q11.2) is also referred to as Ph-positive ALL, the derivative chromosome 22 being known as the Philadelphia (Ph) chromosome. Most cases occur *de novo* but therapy-related cases are also recognized [149,291]. FISH analysis shows that the *BCR-ABL1* fusion gene, formed as a result of the t(9;22) translocation, is present in myeloid cells as well as lymphoid, indicating that the mutation responsible occurs in a pluripotent stem cell [441].

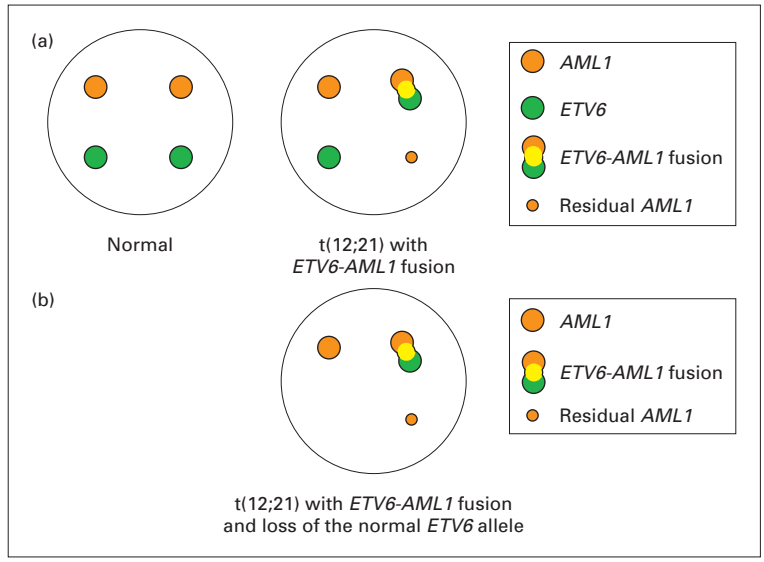


Fig. 3.65 Diagrammatic representation of extra-signal, dual-colour FISH for detection of *ETV6-RUNX1* (*AML1*) fusion in cryptic t(12;21)(p13;q22), using an orange *RUNX1* (*AML1*) probe and a green *ETV6* probe. (a) In a normal cell there are two orange signals and two green signals whereas in a cell with t(12;21) there is one orange *RUNX1* signal, one green *ETV6* signal, a yellow fusion *ETV6-RUNX1* signal and an extra small orange residual *RUNX1* signal. (b) When a translocation is present and the normal allele of *ETV6* has been lost the normal green *ETV6* signal is lacking.

Clinical and haematological features The prevalence of Ph-positive ALL increases steadily with increasing age [409,442] (Fig. 3.66). About 15–30% of adults fall into this category in comparison with only 1–2% of children. The WBC and the peripheral blood blast percentage are higher than in other children or adults with ALL [409,443–445]. Cases may have FAB L1 or L2 cytological features but L2 features are more common than in ALL in general. In two series 70% and 82% of cases, respectively, were classified as L2 [444,446]. Rare cases have L3 cytological features [447]. Occasional cases have an increased basophil count (Fig. 3.67).

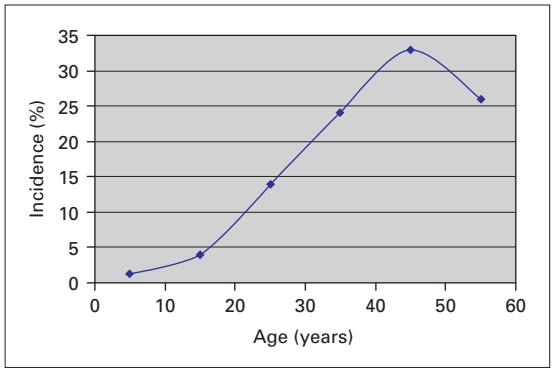


Fig. 3.66 Graph showing the rising incidence of Ph-positive ALL with age.

There is a significant association with the presence of micromegakaryocytes but this observation is not pathognomonic of Ph positivity [448].

With conventional chemotherapy the prognosis has generally been found to be poor, although it is better in children aged between 1 and 9 years than in adolescents and better in adolescents than in adults; in some but not all studies prognosis is improved with more intensive chemotherapy [444,449]. A WBC of below 50×10^9/l has been associated with a better prognosis [450]. The complete remission rate is lower than in other cases of ALL [449] and the subsequent relapse rate is also higher so that both overall and disease-free survival are considerably worse. In one UK trial in children, 5-year event-free survival was only 16% [451] but in a meta-analysis of 10 large series of child and adolescent patients event-free survival was rather better at 31% [452]. In a later UK trial 3-year event-free survival was 52% [450] and in a Nordic trial 10-year survival was 53% [408]. In one large series of adults, 3-year survival was only 19% [453,454] and, in another, 5-year survival was 15% [436]. In a large group of adults ($n = 247$) the 5-year overall survival was 22% [436]. It is possible that the prognosis in children is improved by stem cell transplantation in first remission [452,455] and by the use of daunorubicin [450]. Allogeneic stem cell transplantation has been considered the treatment of choice

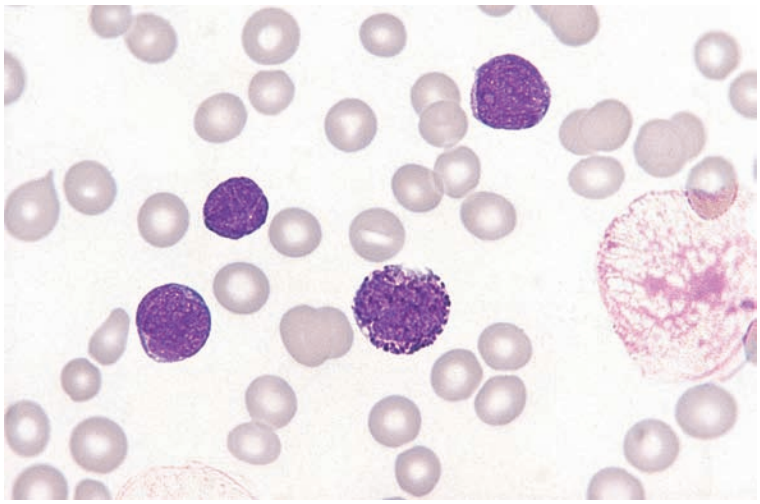


Fig. 3.67 PB film from a patient with FAB L1 Ph-positive ALL showing blast cells and a basophil. MGG ×100.

in adults [453]; this was advised particularly for patients with a WBC of greater than 100×10^9/l, in whom the survival with chemotherapy is extremely poor (5% 5-year survival in one British trial).

The advent of imatinib has led to an improvement in prognosis, e.g. 58% 2-year overall survival in a series with frequent use of haemopoietic stem cell transplantation in addition to imatinib-containing chemotherapy [456]. Imatinib plus corticosteroids is of benefit in elderly patients who, in one study, achieved a median survival of 20 months [457].

Immunophenotype The immunophenotype is usually that of common ALL (about 78% of cases) but a minority have a pre-B immunophenotype (about 20%) and a small minority (about 2%) have an early precursor-B-cell phenotype [404,445,449, 458,459]. In different series of patients, the frequency of detection of myeloid antigens such as CD13 and CD33 has ranged from 20% to 75% [403,444,458,460]. In two series of patients, CD34 was more often expressed than in ALL in general [446,461] but this was not so in another series [444]. CD25 is often expressed [462]. Homogeneous expression of CD10 and CD34 with low but heterogeneous expression of CD38 and expression of CD13 has been found to be reasonably sensitive and specific for *BCR-ABL1*-positive ALL [463]. The myeloid antigen, CD66c, is often positive [54]. Monoclonal antibodies that detect Ph-positive cells with a high sensitivity have been produced [464]; these have been found to be directed at CD66c, which is also expressed in ALL associated with high hyperdiploidy [196].

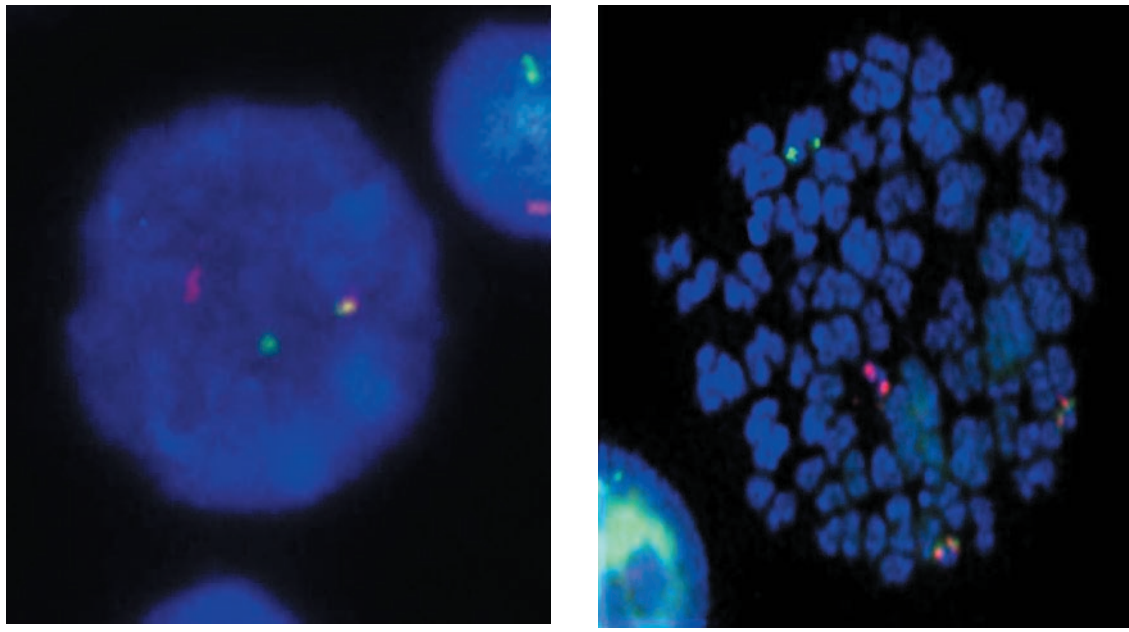
Cytogenetic and molecular genetic features t(9;22) is also the characteristic cytogenetic abnormality of CGL. The translocations in ALL and CGL do not differ cytogenetically but at a molecular level the breakpoint on chromosome 22 may differ. Between 25% and 30% of cases of Ph-positive ALL have an M-BCR breakpoint, i.e. they have the same molecular lesion as occurs in CGL. However, the majority of cases, 70–75%, have an m-BCR breakpoint, this abnormality being very rare in CGL.

The mechanism of leukaemogenesis is fusion of part of the *ABL1* oncogene from chromosome 9 with part of the *BCR* gene on chromosome 22 to form a hybrid gene on chromosome 22 designated *BCR-ABL1*. *ABL1* is homologous with v-*abl*, a retroviral oncogene, which has a role in murine leukaemia. *BCR-ABL1* encodes a chimeric protein with aberrant tyrosine kinase activity, which functions in intracellular signalling pathways. In the majority of cases (about two thirds) of Ph-positive ALL, those with an m-BCR breakpoint on chromosome 22, the BCR-ABL1 protein has a molecular weight of 190 kD while in a minority (about one third), those with an M-BCR breakpoint, it has a molecular weight of 210 kD, as in CGL. In less than 1% of Ph-positive patients, both transcripts are

present [459]. There are only trivial haematological differences between patients with p210 and p190; expression of CD34 may be more frequent in the latter group [465]. Prognosis was better in those with an M-*BCR* breakpoint (p210) in one study [453] but not in another [409]. The prevalence of the two molecular variants varies with age, with cases with a 190 kD transcript increasing from adolescence and those with a 210 kD transcript from early adult life [459].

Characteristic secondary cytogenetic abnormalities in Ph-positive ALL, seen in more than 25% of patients, are duplication of the Ph chromosome (present in about a quarter of patients) [423], abnormalities of 9p (e.g. del(9p), often with loss of the *ABL1-BCR* gene), trisomy 21 and high hyperdiploidy [405,443,456,458,466] (Fig. 3.68). Secondary abnormalities seen in 10–20% of patients are monosomy 7, trisomy 8 and plus X [456,466]. A complex karyotype (at least two extra abnormalities) was seen in half the patients in one study [466]. The presence of secondary cytogenetic abnormalities and of a complex karyotype is associated with a worse prognosis [456,466]. Monosomy 7 as a secondary abnormality is associated with an M-BCR breakpoint, with coexpression of myeloid antigens and with a particularly bad prognosis. Secondary abnormalities of 9p are associated with m-BCR breakpoints, with lack of expression of myeloid antigens and with a very bad prognosis. The prognosis appears to be somewhat better in cases with hyperdiploidy or +der(21) as an associated abnormality [443].

The cytogenetic defect is detectable by a variety of FISH techniques (see page 267). The molecular defect can be detected by RT-PCR. FISH studies show that, as for CGL, there may be loss of chromosome 22 material and, particularly, chromosome 9 material from the der(9) in Ph-positive ALL but the prevalence is much lower than in CGL; this loss of chromosomal material can be seen in patients with an m-*BCR* as well as in those with an M-*BCR* breakpoint [467]. FISH permits the detection of *BCR-ABL1* fusion with a normal karyotype (representing



(a) (b)

Fig. 3.68 FISH in a patient with hyperdiploid *BCR-ABL1*-positive ALL using a dual-colour, single-fusion technique with *BCR* and *ABL1* probes: (a) cell in interphase showing a *BCR* signal (pink), an *ABL1* signal (green) and a *BCR-ABL1* fusion signal (red–yellow–green); (b) cell in metaphase showing the same normal and fusion signals and revealing that the cell is hyperdiploid with two fusion signals. (By courtesy of Dr Magda Jabbar Al-Obaidi, London.)

about a sixth of cases) and also the rare cases due to insertion of *ABL1* into *BCR* [423]. RT-PCR can be used to monitor MRD, the detection of which is of prognostic significance [468].

PAX5 deletion occurs as a secondary genetic event in about half of patients. An even more common second genetic defect is deletion of mutation of the *IKZF1* gene, encoding the lymphoid transcription factor, IKAROS [469]. *IKZF1* is deleted or mutated in more than 80% of patients and, since this is also associated with a poor prognosis in other types of ALL, may be responsible for the poor prognosis of *BCR-ABL1*-positive ALL.

B lymphoblastic leukaemia/lymphoma with t(4;11)(q21;q23); MLL-MLLT2

In the WHO classification this subtype of ALL is grouped with cases with other rearrangements of the *MLL* gene. However, since they differ in some characteristics they are dealt with separately here. ALL with t(4;11)(q21;q23) occurs at all ages but is particularly frequent among babies with congenital ALL and in young infants [211,470]. It constitutes more than half of these cases. Cases occurring in infants often originate in intrauterine life [399,471]; exposure to topoisomerase II-interactive agents during gestation is suspected as an aetiological factor. One percent of children with ALL above the age of 1 year have t(4;11) [411] whereas in adult ALL the prevalence is 3–5% and increases with age [405,449].

Clinical and haematological features Marked splenomegaly and a high WBC are common as is central nervous system disease. Among infants and adults, females are affected more than males, but in the age group 1–14 years males are more affected. Cytological features are of either FAB L1 or L2 ALL but L2 morphology is more common than in ALL in general. Prognosis is very poor in infants and adults but somewhat better in children. In a UK series, the 5-year event-free survival in children with t(4;11) or other translocations involving 11q23 was 30% [451]. In a Japanese series, the 3-year event-free survival was 34% [472]. In a US series of infants under 1 year of age, the 5-year event-free survival was 29% and was even worse in those aged less than 6 months than in older infants [473]. The survival of infants with 11q23 rearrangement appears to be worse than that of other infants with ALL but the survival of infants with t(4;11) appears to be no worse than the survival of infants with other *MLL* rearrangements [472,474], or at least no worse than when there is t(9;11) or t(11;19) [473]. In older children, t(4;11) and t(9;11) are associated with worse outcome than other 11q23 rearrangements [474]. In adults, t(4;11) is associated with an adverse prognosis [409] but this is not so of patients with other 11q23 breakpoints, such as t(11;19) [409]. This subtype of ALL is particularly responsive to cytarabine [475]. Any type of transplantation has been associated with worse outcome than other therapeutic approaches in t(4;11)-associated ALL [474].

Immunophenotype t(4;11)(q21;q23) is strongly associated with early B precursor (pro-B) ALL, i.e. there is positivity for TdT and pan-B markers such as CD19 but CD10 and CD24 are negative. Aberrant expression of CD15 and CD65 is common and CD33 is sometimes positive [476]. CD1d may be expressed [477]. Myeloid antigens are expressed in about half of cases [404]. MPO messenger ribonucleic acid (mRNA) and protein may be expressed [478]. At relapse the immunophenotype is sometimes that of mixed phenotype acute leukaemia or of monoblastic AML. Positivity with a monoclonal antibody (McAb) to chondroitin sulphate proteoglycan, NG2, has been demonstrated [476]. CD133 was consistently expressed in a small number of patients tested, whereas it was expressed in less than half of other patients with ALL [128].

Cytogenetic and molecular genetic features The molecular mechanism of leukaemogenesis in association with t(4;11)(q21;q23) (Fig. 3.69) is formation of a fusion gene, *MLL-MLLT2*, incorporating part of the *MLL* (myeloid–lymphoid leukaemia) gene at 11q23 and part of the *MLLT2* gene (previously known as *AF4*) from 4q21 [396]. *MLLT2* encodes a protein that is probably a transcription factor [479]. *FLT3* is overexpressed in this subtype of leukaemia and in other subtypes associated with *MLL* rearrangement [412]. Prognosis in *MLL*-rearranged infant ALL is worse if there is high level expression of FLT3 [480].

Rearrangement of the *MLL* gene, including that of t(4;11), can be detected by dual-colour, break-apart FISH with a 5′ *MLL*–3′ *MLL* probe. In about a third of patients with 11q23 translocations there is loss of 3′ *MLL* and therefore loss of one colour of the signal. *MLL-MLLT2* fusion can be detected by

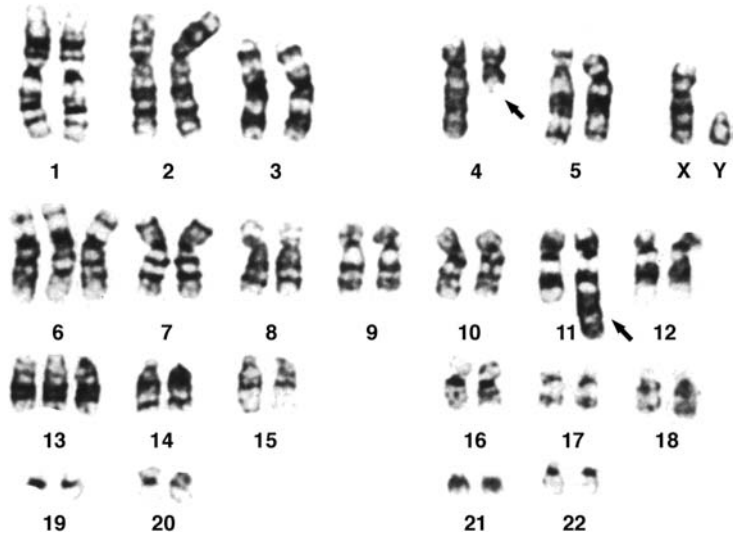


Fig. 3.69 A karyogram showing t(4;11)(q21;q23). (By courtesy of Professor Harry Smith, Brisbane.)

RT-PCR. MRD, which is of prognostic significance, can be monitored by RT-PCR [468]. Rapid reversion to negativity and persistence of negativity for more than 3 months is predictive of a better outcome [481]. Only about 50% of patients have rearrangements of antigen-receptor genes and such rearrangements are therefore often not useful for MRD monitoring [482].

Other B lymphoblastic leukaemia/lymphoma with t(11;v)(q23;v); MLL rearrangement

Other translocations with 11q23 breakpoints and rearrangement of the *MLL* gene are also associated with B-lineage ALL and, sometimes, with MPAL or AML. Some of these are shown in Tables 3.3 and 3.4. Among adults these cases comprise about 4% of cases of ALL [405] and in children 2–3% [403]. Although leukaemias associated with these translocations have some features in common they differ in other characteristics.

Clinical and haematological features The clinical features resemble those of cases associated with t(4;11) in that there is a preponderance of infants and children and an association with a high WBC and central nervous system disease. One neonate with congenital leukaemia associated with t(11;19)(q23;p13) has been reported [231]. If the *MLL* gene is rearranged the prognosis is equally poor in all translocations with an 11q23 breakpoint in some [403,472,483] but not all [474] series. Pui *et al.* found an equally dismal prognosis with all 11q23 rearrangements in infants under the age of 1 year but above this age t(4;11) and t(9;11) appeared to be associated with a worse prognosis than t(11;19) and other rearrangements [474]. As for ALL associated with t(4;11), the prognosis may be better in those between the ages of 1 and 10 years than in infants or older children [455].

Immunophenotype The immunophenotype is usually that of early B precursor (pro-B) ALL. Positive reactions with a McAb to chondroitin sulphate proteoglycan are observed in cases with t(11;19) as well as cases with t(4;11) [476]. Cases with t(11;19)(q23;p13) may likewise show aberrant expression of CD15 and CD65 [476]. The combination of homogeneous expression of CD4, expression of CD56 and lack of expression of CD34 shows a significant correlation with a rearranged *MLL* gene [484].

Cytogenetic and molecular genetic features The molecular mechanism is the fusion of part of the *MLL* gene with one of a number of structurally different genes on a large number of partner chromosomes, some of which are shown in Tables 3.3 and 3.4. Partner chromosomes have included 1, 6, 9, 10, 12, 19, 20 and X [212–214,396]. Cases that have cytogenetic abnormalities with 11q23 breakpoints but without *MLL* rearrangement, e.g. many cases with

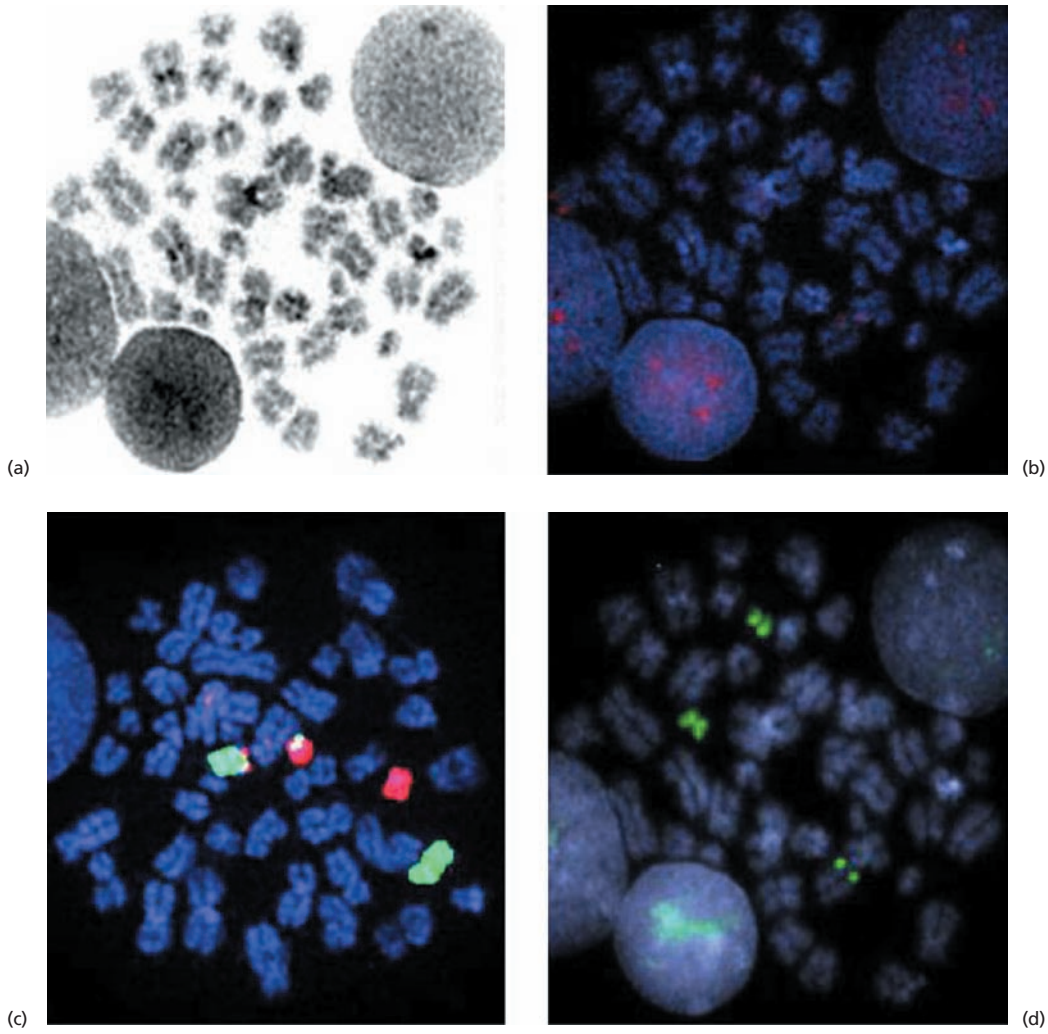


Fig. 3.70 A subtle t(11;19)(q23;p13.3) demonstrated by FISH: (a) metaphase spread; (b) rearrangement of *MLL* demonstrated with a *MLL* probe (pink) which has been split so that there are three signals instead of two; (c) whole chromosome paints for chromosome 11 (green) and chromosome 19 (pink) showing that these two chromosomes are involved in the rearrangement; (d) whole chromosome paint for chromosome 19 (green) showing signals on the normal 19, derivative 19 and derivative 11. (By courtesy of Dr Magda Jabbar Al-Obaidi.)

del(11)(q23), have different disease characteristics including a better prognosis and are not included in this subtype of ALL [483].

Rearrangements of the *MLL* gene can be detected by dual-colour, break-apart FISH and with whole chromosome paints (Fig. 3.70).

Many of the molecular rearrangements resulting from translocations with an 11q23 breakpoint can be demonstrated by RT-PCR and rearrangement of the *MLL* gene can be demonstrated by Southern blotting.

B lymphoblastic leukaemia/lymphoma with t(1;19)(q23;p13); TCF3-PBX1

This subtype constitutes 2–5% of childhood ALL and 1–3% of adult cases [403,405,449]. The

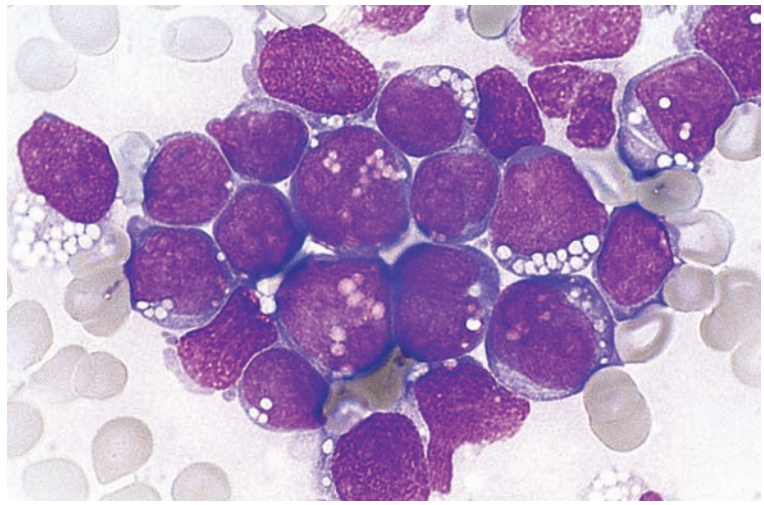


Fig. 3.71 BM film in ALL with t(1;19)(q23;p13) and FAB L3 cytological features. MGG ×100. (By courtesy of Dr Georges Flandrin, Paris.)

translocation was first recognized by Carroll and colleagues in 1984 [485]. A minority of childhood cases are of intrauterine origin [399,486]. Diagnosis of this subtype of ALL can lead to an adjustment of methotrexate therapy.

Clinical and haematological features Adult patients are relatively young [403]. In one series there was a correlation with a high WBC, non-white ethnic origin and central nervous system disease [487]. However, in other series of adult cases the WBC was generally low [404,405]. Cytological features are most often FAB L1 but sometimes L2 and sometimes L3 [488] (Fig. 3.71). The cytological features can change from L1 to L3 with disease progression [489]. The prognosis in childhood cases was previously poor. However, with current more intensive treatment, outcome in several series of patients has been as good as, if not better than, that of ALL associated with high hyperdiploidy [403,408,490]. A poor prognosis has been reported in adults [491]. Because of the greater risk of central nervous system disease and the biological features of the leukaemic cells, an increased dose of methotrexate may be indicated [492]. Leucoencephalopathy occurring during complete remission has been reported, in a number of studies, to be more common in this subtype of ALL [493].

Immunophenotype The immunophenotype is often that of pre-B ALL, i.e. cytoplasmic μ chain is present but not surface membrane immunoglobulin. About a quarter of cases with a pre-B immunophenotype are found to have this translocation. The typical immunophenotype is CD19, CD22, CD10 and CD9 positive and CD21 and CD34 negative. CD20 expression may be positive or negative [487] or there may be both positive and negative blast cells. CD45 is strongly expressed. This immunophenotype is characteristic but not specific [196]. A smaller number of cases have a common ALL phenotype (CD10 positive, cytoplasmic μ chain negative) or express both surface and cytoplasmic immunoglobulin. If a smaller range of McAb is used, positivity for CD19 and CD10 with negativity for CD34 is suggestive of t(1;19) and can be taken as an indication for cytogenetic or molecular genetic analysis [494]. Polyclonal [495] and monoclonal [496] antibodies to the TCF3-PBX1 fusion protein (see below) have been used successfully in the identification of t(1;19)-associated ALL. The monoclonal antibody is applicable to both flow cytometry and immunohistochemistry [496].

Cytogenetic and molecular genetic features The molecular mechanism of leukaemogenesis is fusion of the *PBX1* gene from 1q23 with part of the transcription activator gene, *TCF3* (previously *E2A*), at 19p13 to form a hybrid *TCF3-PBX1* gene, which encodes an abnormal transcription factor [497]. There is also aberrant expression of the *MER* gene, which encodes MERTK receptor tyrosine kinase [498].

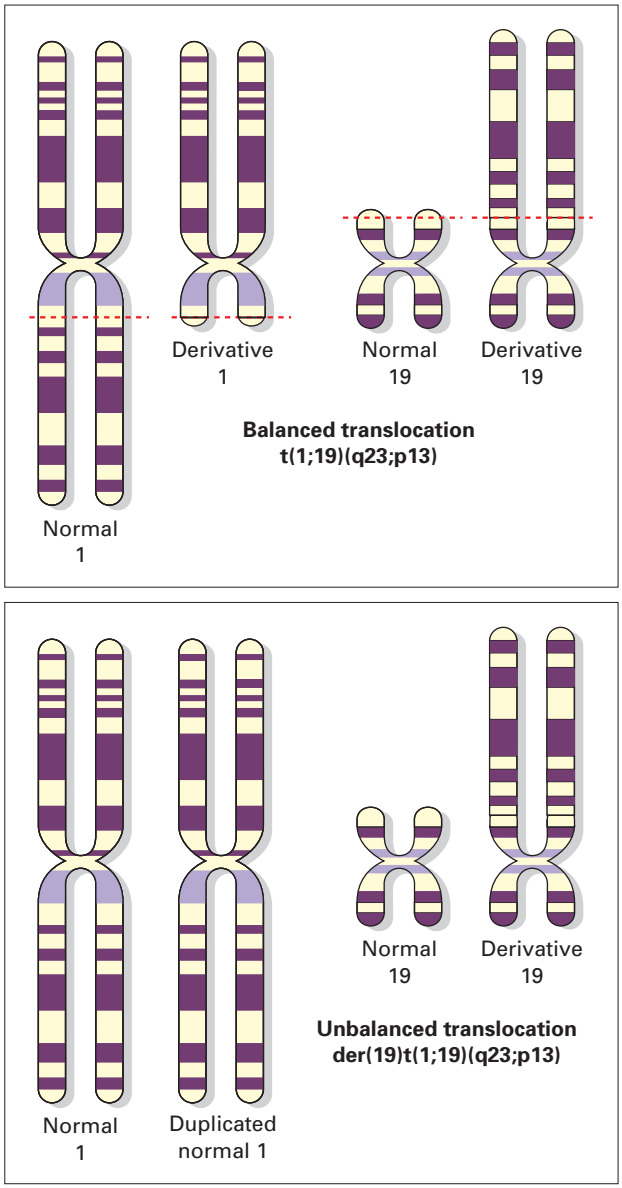


Fig. 3.72 Diagrammatic representation of balanced and unbalanced forms of t(1;19)(q23;p13).

ZAP70 is overexpressed, as it is in other cases of ALL with a pre-B immunophenotype [499].

t(1;19) may occur as a balanced or unbalanced translocation. A straightforward balanced translocation, t(1;19)(q23;p13), is the less common abnormality. More common is the unbalanced der(19)t(1;19)(q23;p13) in which the derivative chromosome 1 has been lost and has been replaced by a second copy of the normal chromosome 1 (Fig. 3.72). There is a prognostic difference between the balanced and the unbalanced translocation, the unbalanced cases having a better prognosis in three series of patients [455,490,500].

t(17;19)(q21–22;p13) is a molecular variant of t(1;19), which leads to fusion of *TCF3* with *HLF* (hepatic leukaemia factor). The fusion gene, *TCF3-HLF*, encodes an abnormal transcription factor. The prognosis is very poor.

A double-colour, break-apart FISH strategy has been devised which both detects t(1;19) and

distinguishes balanced and unbalanced translocations [501]. In addition, it is abnormal in patients with t(17;19)(q21–22;p13). The molecular defect of both t(1;19) and t(17;19) can be detected by RT-PCR. RT-PCR can be used for the detection and monitoring of MRD in patients with t(1;19). However, prolonged detection of MRD has been found to be compatible with continuing complete haematological remission [481].

B lymphoblastic leukaemia/lymphoma with t(5;14)(q31;q32); IGH-IL3

This is a rare form of B-lineage ALL but is important because it may be confused with eosinophilic leukaemia if not adequately investigated.

Clinical and haematological features The features are those of ALL but with marked elevation of the eosinophil count as a result of dysregulation of the *IL3* gene. The eosinophils are not part of the leukaemic clone. They may, however, show cytological abnormalities such as hyperlobulation and degranulation [502] (Fig. 3.73). Because of the large number of eosinophils and precursors in the bone marrow, the blast percentage may not be very high.

Immunophenotype The immunophenotype is that of common ALL.

Cytogenetic and molecular genetic features The mechanism of eosinophilia is dysregulation of *IL3*. The mechanism of leukaemogenesis has not been defined.

B lymphoblastic leukaemia/lymphoma with hypodiploidy

This is an uncommon form of B-lineage ALL (1–5% of cases). Hypodiploidy can be further categorized as near haploidy (23–29 chromosomes), low hypodiploidy (33–39 chromosomes) and high hypodiploidy (42–45 chromosomes) [503]. Near haploid metaphases usually retain both sex chromosomes and preferentially gain chromosomes 10, 14, 18 and 21 into the haploid chromosome set [504]. Low hypodiploidy and near triploidy are considered to represent the same subgroup and subclones of both types may coexist [505]. Patients with hypodiploidy with 23–29 chromosomes have a median age of 7 years whereas those with hypodiploidy with 33–39 chromosomes have a median age of 15 years [503]. Both tend to have a low WBC (less than 50×10^9/l). Both may have an associated hyperdiploid clone as a result of endoreduplication. A considerably worse prognosis than is seen in ALL in general has been reported in hypodiploidy with 33–44 chromosomes [506], in near haploidy (whether defined as 23–29 or as 24–28 chromosomes) [429,503,506], in patients with 33–39 chromosomes [503] and in patients with 30–39 chromosomes [505]. In one study, patients with more than 40 chromosomes – including adults as well as children – appeared to have an intermediate prognosis [503]. In another study, patients with 24–29, 33–39 and 40–43 chromosomes had a similar adverse prognosis but those with 44 chromosomes had a significantly better outcome [507]. Such cases can result from monosomy or from unbalanced translocations leading to formation of dicentric chromosomes, particularly dic(9;20)(p11~p13;q11).

In the WHO classification hypodiploidy is defined by having less than 46 chromosomes but with the comment that it might be better defined by less than 45 or less than 44 chromosomes. Prognosis is poor.

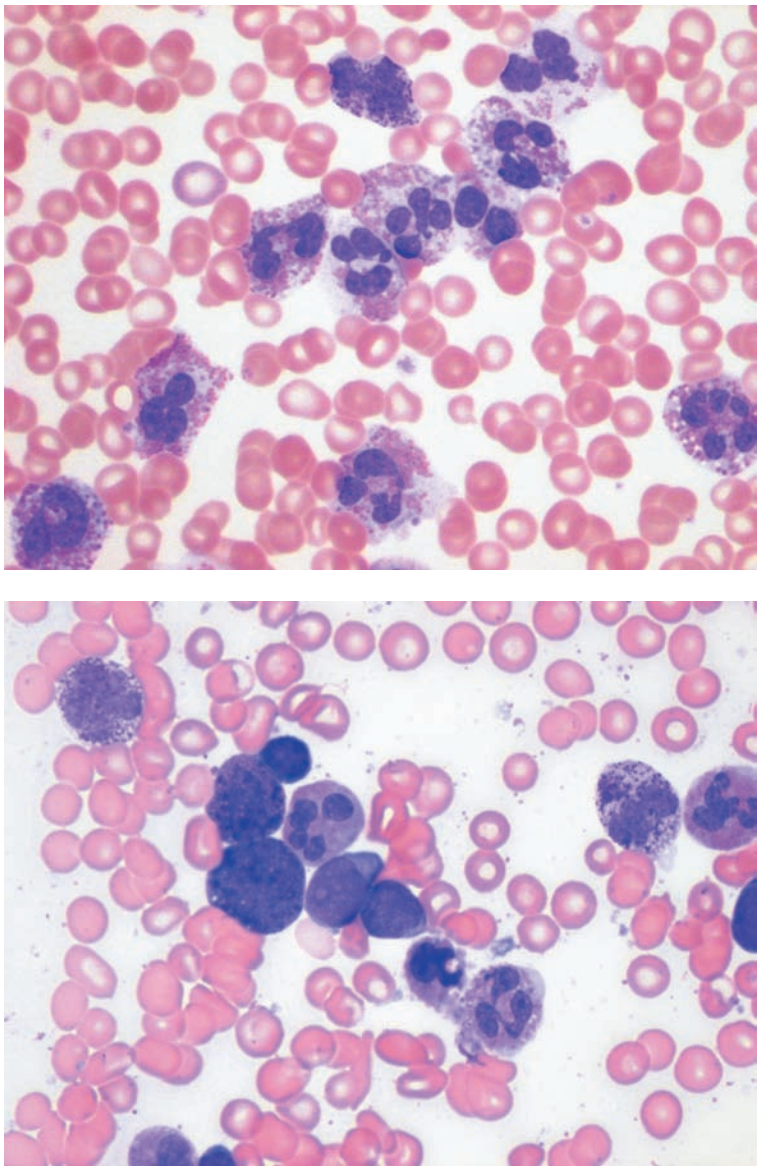
Clinical and haematological features There are no specific features recognized.

Immunophenotype The immunophenotype is usually that of common ALL.

Cytogenetic and molecular genetic features There may be associated structural chromosomal abnormalities. *PAX5* deletions are usually present, often with an associated point mutation in the other allele. Because of the poor prognosis, screening of all patients with failed or normal cytogenetic analysis for near haploidy and for low hypodiploidy is advised [503,504]. The DNA index is low. DNA quantification histograms also permit detection of a prognostically important small hypodiploid clone.

Other genetic subtypes that have not yet been incorporated into the WHO classification of B lymphoblastic leukaemia/lymphoma

A large number of other cytogenetic/genetic abnormalities have been described in B-lineage ALL. Some of these are summarized in Table 3.10 and the FISH diagnosis of one of these subtypes is illustrated in Fig. 3.74. In the WHO classification these are assigned to the category of B lymphoblastic



(a)

(b)

Fig. 3.73 ALL with eosinophilia associated with t(5;14)(q31;q32). (a) PB film showing hypereosinophilia with a minor degree of eosinophil degranulation and vacuolation. MGG ×100. (b) BM film showing abnormal eosinophils and lymphoblasts. MGG ×100. (By courtesy of Dr James Vardiman, Chicago.)

leukaemia/lymphoma, not otherwise specified. Complex karyotypes (five or more aberrations), seen in about 5% of adult patients, are associated with a worse prognosis [505].

The t(14;18)(q32;q21) translocation that characterizes follicular lymphoma can also occur in B-lineage neoplasms with blastic morphology (FAB L1, L2 or L3) [510,511]. When the immunophenotype is of pre-B or common ALL classification as ALL is clearly appropriate whereas cases that are morphologically blastic but immunophenotypically mature [512] are probably better regarded as non-Hodgkin lymphoma.

B-lineage ALL is 10–20 times more common in children with Down syndrome and has specific genetic characteristics. There is an association with +X as an isolated acquired abnormality and an increased prevalence of t(8;14)(q11;q23) with dysregulation of *CEBPD* by proximity to *IGH* [411]. A mutation in the pseudokinase domain of *JAK2* is

Table 3.10 Some of the less common genetic categories of B-lineage acute lymphoblastic leukaemia [200,212–214,411,418,508,503,505,507–509].

Translocation or other non-random abnormality	Type of leukaemia	Molecular event	Reference
ins(5;11)(q31;q13q23)		*AF5q31-MLL*	
t(6;11)(q27;q23)	Early-B-cell precursor ALL, AML, T-ALL	*MLL-MLLT4* (*AF6*)	[212]
t(9;11)(p21;q23)	Early-B-cell precursor ALL, AML, MPAL	*MLLT3-MLL*	[200]
t(10;11) (p12–22;q23)	Early-B-cell precursor ALL	*MLL-MLLT10* (*AF10*)	[213]
t(11;19) (q23;p13.3)	Early-B-cell precursor ALL, common and pre-B ALL, AML, T-ALL	*MLL-MLLT1* (*ENL*)	[214]
t(X;11)(q13;q23)	ALL	*MLL-MLLT7* (*AFX1*)	
dic(7;12)(p11;p12)	Common or pre-B ALL	Unknown	
t(8;14)(q11.2;q32)	Pre-B ALL, often in Down syndrome	*IGH* gene rearranged	[411]
dic(9;12) (p11-12;p11-12)	Common ALL	Unknown*	
dic(9;20)(p11-13;q11)	Common or pre-B ALL	Unknown	
t(14;18)(q32;q21)	Common or pre-B ALL	*IGH-BCL2*	
t(14;19)(q32;q13)	B lineage ALL		[503]
inv(19)(p13q13) (cryptic)		*TCF3-FB1* fusion	[508]
Internal amplification of chromosome 21 sequences	Common and pre-B ALL	Amplified sequences on 21, *LGMN* overexpressed	[505,509]
t(14;19)(q32;q13)		*CEBPA* upregulated	
t(14;19)(q32;q13)		*CEBPG* upregulated	
t(8;14)(q11;q32)		*CEBPD* upregulated	
t(14;14)(q11;q32)		*CEBPE* upregulated	
inv(14)(q11q32)		*CEBPE* upregulated	
t(14;20)(q32;q13)		*CEBPB* upregulated	

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; MPAL, mixed phenotype acute leukaemia.
* An *ETV6-RUNX1* transcript was detected in 4 of 4 cases tested suggesting that a cryptic t(12;21)(p13;q22) was also present [418].

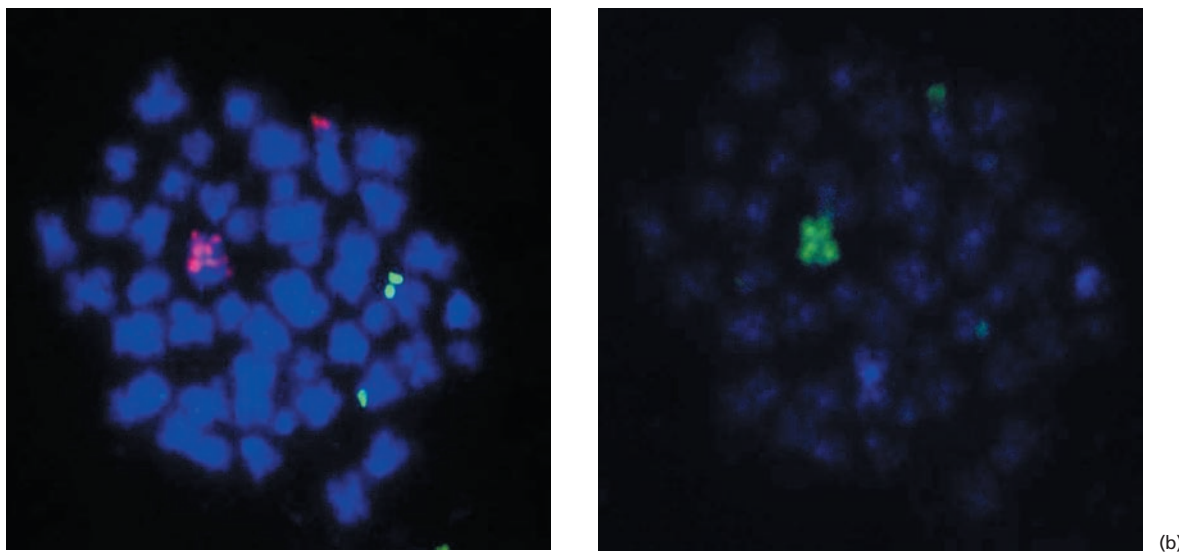


Fig. 3.74 Demonstration of internal amplification of chromosome 21q sequences (iAMP21) demonstrated by FISH: (a) using probes for *RUNX1* (pink) and *ETV6* (green) (a probe pair used for detection of *ETV6-RUNX1* fusion); the *ETV6* signals on chromosome 12 are normal while the *RUNX1* signals are normal on one chromosome 21 but amplified on the other; (b) whole chromosome paint for chromosome 21 demonstrating that the amplified signals seen in (a) are on chromosome 21. (By courtesy of Dr Magda Jabbar Al-Obaidi.)

present in about a fifth of patients and appears to be specific to ALL associated with Down syndrome.

B lymphoblastic leukaemia/lymphoma, not otherwise specified

This category includes all cases of B-lineage ALL/lymphoblastic lymphoma that have not been assigned to specific categories in the WHO classification [513]. It is necessarily a heterogeneous group.

Clinical and haematological features There are no specific clinical features recognized. Patients are often children. Cytological features are those of FAB L1 or L2 ALL. In cases designated ALL there are almost always more than 20% bone marrow blast cells. Patients with a tissue mass with no bone marrow infiltration or with less than 25% bone marrow blast cells can be considered to have lymphoblastic lymphoma. Extramedullary disease (liver, spleen, lymph nodes, central nervous system and testis) is common but presentation as lymphoblastic lymphoma is infrequent.

Immunophenotype The immunophenotype is variable. There is generally expression of CD19, CD79a and cytoplasmic CD22 and there may be expression of CD10, CD20, membrane CD22, CD24, PAX5, CD34 and TdT. CD45 may be weak or negative.

Cytogenetic and molecular genetic features Some patients will have the recurrent genetic abnormalities shown in Table 3.10. Others have less specific clonal abnormalities such as del(6q), del(9p) or del(12p). Deletion of *PAX5* is found in about a third of patients. Deletion or mutation of *IKZF1*, encoding IKAROS, is a recurrent genetic abnormality in high risk disease and is an independent predictor of worse outcome [469].

T lymphoblastic leukaemia/lymphoma

T acute lymphoblastic leukaemia constitutes only 15% of cases of childhood ALL whereas among cases of lymphoblastic lymphoma, T-lineage disease is considerably more common than B-lineage, comprising about 90% of cases. Typically T-ALL occurs at an older age than in B-lineage disease. There is a male predominance. There is only a weak relationship between immunophenotype and specific cytogenetic abnormalities and neither correlates with the FAB morphological type. The 2008 WHO classification does not further categorize T-lineage ALL/lymphoblastic lymphoma [514].

Clinical and haematological features Primary involvement may be of the bone marrow and blood or of the thymus or other extranodal site. There are some clinical and biological differences between cases presenting as ALL and cases presenting as lymphoblastic lymphoma. In ALL there tends to be more hepatosplenomegaly and less mediastinal involvement, the immunophenotype is often more immature and the gene expression profile differs [515].

The WBC is typically high but, in comparison with B-lineage disease, bone marrow function is often better preserved. Cytological features are usually FAB L1 or L2 ALL (rarely L3). There are usually at least 20% bone marrow blasts in cases categorized as ALL. The distinction between ALL and lymphoblastic lymphoma is, to some extent, arbitrary, but for clinical trial purposes the presence of a soft tissue mass with less than 25% bone marrow blast cells is often used as a criterion for a diagnosis of lymphoblastic lymphoma.

Immunophenotype The defining feature is expression of cytoplasmic CD3. Other immunophenotypic markers are discussed on pages 89–90.

Cytogenetic and molecular genetic features Cytogenetic abnormalities may be non-specific (e.g. 6q– or 9p–) but about a quarter of cases involve the *TCR* loci, particularly the *TCRA* and *TCRD* loci at 14q11.2 and the *TCRB* locus gene at 7q35 but occasionally the *TCRG* locus at 7p15-14. Details of these and other genetically defined categories are shown in Table 3.11 and representative abnormalities are discussed in more detail below.

In addition, one deletion and several point mutations are likely to be important in pathogenesis [514]; these occur in all the major genetic subtypes. About 30% of patients have del(9p) leading to loss of the tumour suppressor gene, *CDKN2A*. Activating mutations in *NOTCH1* are found in about 50% of patients [523], and another 30% of patients have a mutation in *FBXW7*. *NOTCH1* encodes a membrane receptor that regulates normal T-cell development and FBXW7 reduces NOTCH1 activity.

Table 3.11 Some genetic categories of T-lineage ALL. (Derived from references 403, 514 and 516–522, and other sources.)

Cytogenetic abnormality	Mechanisms of leukaemogenesis	Approximate frequency
t(1;7)(p34;q34)	*LCK* gene at 1p34 dysregulated by proximity to the *TCRB* locus	3%
t(1;14)(p32;q11) t(1;7)(p32;q35)	*TAL1* (*SCL*) gene at 1p32, a gene encoding a TF, is dysregulated by proximity to the *TCRAD* locus at 14q11.2 or the *TCRB* locus at 7q35	<1%
cryptic *TAL*[d]	*SIL-TAL1* fusion resulting from a cryptic deletion at 1p32	20–30% [516]
t(5;14)(q35;q32) (cryptic)	*TLX3* (*HOX11L2*) dysregulation, probably by proximity to *CTIP1*	20% [517]
t(6;11)(q27;q23)	*MLL-MLLT4* (*AF6*) fusion	<1%
t(7;9)(q34;q34.3)	*NOTCH1* (*TAN1*), a gene encoding a transmembrane protein active in signal transduction, is disrupted and the truncated protein produced is located in the nucleus	3–5%
t(7;9)(q35;q32)	*TAL2* gene, a TF gene at 9q32, is dysregulated by proximity to the *TCRB* locus at 7q35	<1%
t(7;10)(q35;q24) t(10;14)(q24;q11)	*TLX1* (*HOX11*) at 10q24 dysregulated by proximity to the *TCRB* locus at 7q35 or the *TCRAD* locus at 14q11.2	5–7% of children 14–30% of adults [403,517]
t(7;11)(q35;p13)	*LMO2* (*RBTN2*) at 11p13, a TF gene, is dysregulated by proximity to the *TCRB* locus at 7q35	2–7%
t(7;19)(q35;p13)	*LYL1* gene, a TF gene at 19p13.2-13.1, is dysregulated by proximity to the *TCRB* locus at 7q35	<1%
t(8;14)(q24;q11)	*MYC* at 8q24 is dysregulated by proximity to the *TCRAD* locus at 14q11.2	1–2%
t(9;22)(q34;q11.2)	*BCR-ABL1*, more often m-BCR	Rare [519]
t(10;11)(p12–22;q23)	*MLL-MLLT10* (*AF10*) fusion	Up to 8% in adults [520] <1% [521]
t(10;11)(p13-14;q14-21)	*PICALM-MLLT10* (*AFIO*) fusion	9%, all TCRγδ expressing, of which it is found in 25% [522]
t(11;14)(p15;q11)	*LMO1* (*RBTN1*) at 11p15, a TF gene, is dysregulated, by proximity to the *TCRAD* locus at 14q11.2	
t(11;14)(p13;q11)	*LMO2* (*RBTN2*) at 11p13, a TF gene is dysregulated, by proximity to the *TCRAD* locus at 14q11	<1%
t(11;19)(q23;p13.3)	*MLL-MLLT1* (*ENL*) fusion*	3% [521]
inv(14)(q11q32) t(14;14)(q11;q32)	*TCLA1* (*TCL1*) at 14q32.1 is dysregulated by proximity to the *IGH* locus at 14q32	
t(6;11)(q27;q23)	*MLL-AF6q27* fusion	[520]

m-BCR, minor breakpoint cluster region; TF, transcription factor.
* Good prognosis.

There is some correlation between genetic abnormalities and other features.

Cases expressing *LYL1* and those with an *MLL-MLLT1* fusion are usually CD4-negative CD8-negative CD34-positive pro-T cells while cases with *MLL-MLLT1* are TCR-γδ positive [524]. Cases with *TLX1* or *TLX3* dysregulation have been reported to have an early thymocyte phenotype and *TAL1*-positive cases to have a late cortical thymocyte phenotype [524].

Genetic subtypes of T lymphoblastic leukaemia/lymphoma not yet incorporated into the WHO classification

There are some genetic categories of T-ALL included in Table 3.11 that have not yet been incorporated into the WHO classification but that nevertheless have definable characteristics. Whether these subtypes are of clinical significance is not yet clear. Some of these will now be discussed.

T lymphoblastic leukaemia/lymphoma with t(10;14)(q24;q11); TLX1 dysregulation

This category of ALL comprises about 5–7% of childhood T-lineage ALL and about 14–30% of adult T-lineage ALL [403,517].

Clinical and haematological features The majority of cases have FAB L1 cytological features. Prognosis is relatively good [524].

Immunophenotype The immunophenotype may be early thymocyte [524] or intermediate or common thymocyte with coexpression of CD10 in a quarter of patients [403].

Cytogenetic and molecular genetic features The mechanism of leukaemogenesis is dysregulation of *TLX1* (*HOX11*), a transcription factor gene, as a consequence of proximity to the *TCRAD* locus at 14q11.2. A similar mechanism of leukaemogenesis is operative in T-lineage ALL with t(7;10)(q35;q24) when the *TLX1* gene is dysregulated by proximity to the *TCRB* locus at 7q35. Both rearrangements are detectable by PCR. *TLX1* activation without either of these translocations is associated with similar disease characteristics. *TLX1* expression has been related to a better prognosis [194].

T lymphoblastic leukaemia/lymphoma with t(5;14)(q35;q32) (cryptic); TLX3 dysregulation

This cryptic translocation is found in about 20% of patients with T-lineage ALL.

Immunophenotype The majority of cases express CD1a, CD2, CD4, CD5, CD7 and CD8 [54].

Cytogenetic and molecular genetic features The breakpoint on chromosome 5 is in the region of *TLX3* (*HOX11L2*), which is transcriptionally activated, probably by proximity to the transcription regulatory elements of *CTIP2* at 14q32.1 [517]. The translocation can be detected by dual-colour, break-apart FISH using a probe that spans *TLX3*.

T lymphoblastic leukaemia/lymphoma with cryptic TAL^d deletion; SIL-TAL

This is one of the commoner subtypes of T-lineage ALL, accounting for up to a third of cases. It is more frequent among children and adolescents than adults [516]. The genetic abnormality, a small deletion on chromosome 1, is detectable only by molecular techniques.

Clinical and haematological features Clinical and haematological features have been reported not to differ from other cases of T-lineage ALL [518]. Results in a small series of patients suggested a high WBC and an adverse prognosis [525].

Immunophenotype No specific immunophenotype has been recognized except that this subtype of T-lineage ALL occurs preferentially among cases expressing *TCR*αβ and CD2, whereas CD10 is not expressed [54].

Cytogenetic and molecular genetic features The mechanism of leukaemogenesis is that a small deletion leads to the fusion of most of the sequences of a transcription factor gene on chromosome 1, *TAL1* (*SCL*), with the promoter of an upstream gene, *SIL*. This leads to dysregulation of the *TAL1* gene (which is normally expressed in haemopoietic precursors and endothelial cells but is not expressed in normal T cells). Breakpoints in both the *SIL* gene and the *TAL* gene differ. The *TAL* gene can also be dysregulated by translocations (see Table 3.11) but this is much less common than dysregulation as a consequence of a microdeletion.

Submicroscopic deletion involving 1q32, *TAL^d*, can be detected by PCR or RT-PCR [516]. RT-PCR detects a higher proportion of cases than genomic PCR. A dual-colour, break-apart FISH technique can also be used, employing a red *SIL*–green *TAL1* fusion probe. The red colour is lost from one of the fusion signals in the presence of the deletion. The same probe will detect t(1;14)(p32;q11), one of the fusion signals being split.

T lymphoblastic leukaemia/lymphoma with NUP214-ABL1 fusion

This appears to be a unique mechanism of leukaemogenesis. There is fusion of *NUP214* and *ABL1* and high level amplification within episomes (DNA units that are not detectable on conventional cytogenetic analysis) [526]. It is likely that the gene fusion has occurred on circularized DNA.

Clinical and haematological features Cases are T lineage and it may be predicted that disease is imatinib sensitive.

Immunophenotype Other than being of T lineage no specific features have been identified.

Cytogenetic and molecular genetic features These cases may be identified on FISH analysis using *ABL1* probes when it appears as amplification of *ABL1* [423].

Natural killer cell lymphoblastic leukaemia/lymphoma

NK cell lymphoblastic leukaemia/lymphoma is a rare neoplasm assigned in the WHO classification to 'Other ambiguous lineage leukaemias'. It has been suggested that a significant proportion of cases previously assigned to T-ALL, on the basis of expression of cytoplasmic CD3 and sometimes a mediastinal mass, might represent immature NK cells [527]. There is expression of markers shared with the T lineage, such as CD7 and CD2 and cytoplasmic CD3, but without rearrangement of the *TCR* loci. Cases are provisionally identified by expression of CD94 1A, an isoform of CD94 that is induced in NK cell precursors by interleukin 15; CD56 is not expressed [527].

Therapy-related lymphoblastic leukaemia/lymphoma

Therapy-related ALL, although rare, is being increasingly recognized. Such cases are usually either Ph positive or associated with balanced translocations with an 11q23 breakpoint (see Table 3.6); they follow administration of topoisomerase II-interactive drugs. The latent period is short, usually less than 2 years. Cases associated with 11q23 breakpoints and rearrangement of the *MLL* gene have included t(1;11)(p32;q23), t(4;11)(q21;q23), t(5;11)(q35;q23), t(11;16)(q23;p13) and t(11;19)(q23;p13) [293]. The great majority of cases are of B lineage but one case of T-lineage ALL has been reported associated with *MLL* rearrangement [293].

Conclusions

The classification of acute leukaemia is becoming increasingly complex and this trend is likely to continue. It is, however, apparent that this increasing complexity means that entities with important biological differences are now being recognized. Improvements in treatment outcome have already resulted from a greater understanding of this complexity and such advances are also likely to continue.

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FOUR

THE MYELODYSPLASTIC SYNDROMES

Recognition, nature and epidemiology

The myelodysplastic syndromes (MDS) are a related group of bone marrow disorders that were described in the 1930s and 1940s, using terms such as 'primary refractory anaemia' and 'preleukaemic anaemia' [1]. The first description may date back to 1900 when Leube described, in the German literature, a patient with a macrocytic anaemia that progressed to acute leukaemia [2]. The myelodysplastic syndromes have been increasingly recognized in the last 25–30 years since the French–American–British (FAB) group suggested criteria to identify them and to distinguish them from the acute leukaemias. In the initial FAB classification they were designated the dysmyelopoietic syndromes and were divided broadly into chronic myelomonocytic leukaemia (CMML) (no longer classified as MDS) and refractory anaemia with excess of blasts (RAEB) [3]. RAEB included cases that would previously have been classified not only as 'primary refractory anaemia' and 'preleukaemic anaemia' but also as 'preleukaemia', 'smouldering leukaemia', 'subacute leukaemia' or 'atypical leukaemia'. The dysmyelopoietic syndromes, renamed the myelodysplastic syndromes, were further described and classified by the FAB group in 1982 [4] with the criteria for making a distinction from acute myeloid leukaemia (AML) being further refined by this group in 1985 [5] and by World Health Organization (WHO) expert groups in 2001 and 2008 [6,7].

Overall the incidence of MDS is about three times that of AML [8] and rises exponentially with age. Most published estimates fall between 3 and 12/100 000/year, with higher estimates more likely to be correct [8]. Before the age of 60 years, AML is more common than MDS but above the age of 65 years the incidence of MDS is consistently higher [8]. The

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incidence in men is about twice that in women [8,9] and is higher in white Americans than black or Asian Americans [9].

The MDS are a closely related group of acquired bone marrow disorders characterized by ineffective and dysplastic haemopoiesis. These conditions are intrinsic to the bone marrow and are progressive. They result from the proliferation of an abnormal clone of cells that replaces normal haemopoietic cells. The defect may be principally manifest in one lineage (the granulocytic/monocytic lineage, the erythroid lineage or, less often, the megakaryocyte lineage) but commonly dysplasia is bilinear or trilinear. There is usually a discrepancy between a normocellular or hypercellular bone marrow and peripheral blood cytopenia, although in up to 10% of cases the bone marrow is hypocellular. (It should be mentioned that, since MDS occurs predominantly in the elderly, it is important to interpret bone marrow cellularity in relation to the age of the patient.) Although cytopenia is most characteristic, some patients have neutrophil leucocytosis, monocytosis or thrombocytosis or, rarely, eosinophilia or basophilia.

A number of aetiological factors are known, including irradiation, exposure to anti-cancer chemotherapy (particularly alkylating agents but also topisomerase II-interactive drugs), benzene and cigarette smoking. MDS following benzene exposure has similar characteristics to therapy-related MDS (hypocellularity and abnormalities of chromosomes 5 and 7) [10]. An association with alemtuzumab therapy has also been suspected [11]. There are also genetic disorders that predispose to MDS; these include Diamond–Blackfan anaemia, Fanconi anaemia, Rothmund–Thomson syndrome [12], dyskeratosis congenita, Shwachmann–Diamond syndrome and Bloom syndrome. In addition, acquired clonal and non-clonal haematological disorders may progress to MDS; these include paroxysmal nocturnal haemoglobinuria and aplastic anaemia.

Dysplastic haemopoiesis is not confined to MDS, being seen, for example, during administration of certain drugs, during exposure to various toxic substances such as heavy metals, in copper deficiency, in human immunodeficiency virus (HIV)-infected subjects [13] and in megaloblastic anaemia secondary to vitamin B_{12} or folic acid deficiency. The diagnosis of MDS requires the recognition of features consistent with this diagnosis and either demonstration of clonality or the exclusion of alternative causes or both. Haematological abnormalities that may occur are shown in Table 4.1 [14–26]. The usefulness of such features in diagnosis varies. Some abnormalities, such as an acquired Pelger–Huët anomaly (Fig. 4.1) or micromegakaryocytes (Fig. 4.2), are highly characteristic and almost pathognomonic of MDS; one or other of these abnormalities occurs in a high percentage of patients [27]. Agranular neutrophils (Fig. 4.3) are also highly specific but are present in a smaller proportion of

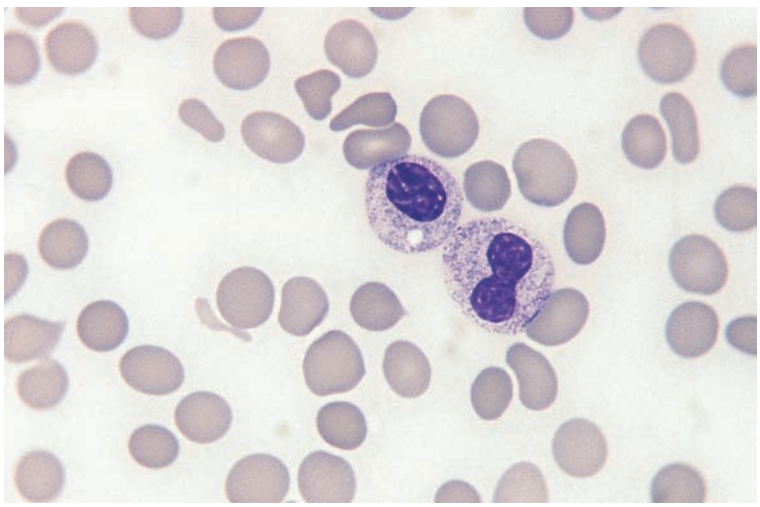


Fig. 4.1 Peripheral blood (PB) film of a patient with therapy-related myelodysplastic syndrome (MDS) showing the acquired Pelger–Huët anomaly; also apparent are anisocytosis, poikilocytosis and severe thrombocytopenia. The bone marrow (BM) showed trilineage myelodysplasia. May–Grünwald–Giemsa (MGG) ×100.

Table 4.1 Haematological features that may occur in the myelodysplastic syndromes (MDS).

Peripheral blood	Bone marrow
Erythropoiesis	
Anaemia and red cell dysplasia	Hyperplasia (common)
Normocytic normochromic (common)	Hypoplasia (uncommon) including red cell aplasia
Macrocytic (common)	Megaloblastic erythropoiesis
Microcytic (uncommon)*	Macronormoblastic erythropoiesis
Dimorphic blood film	Sideroblastic erythropoiesis
Anisocytosis, anisochromasia	Dysplastic erythropoiesis with features such as binuclearity, multinuclearity, nuclear lobulation or fragmentation, increased Howell–Jolly bodies, internuclear bridges, gaps in nuclear membrane, pyknosis, gigantism, megaloblastosis
Poikilocytosis (which may include ovalocytes, elliptocytes† [14–16], schistocytes [14,17], teardrop poikilocytes, stomatocytes, acanthocytes [18] and target cells)	
Polychromasia (uncommon),	
Pappenheimer bodies, basophilic stippling	
Circulating nucleated red blood cells, which may show dyserythropoietic or megaloblastic features or defective haemoglobinization	
Decreased reticulocyte count (usually)	
Increased reticulocyte count (rarely) [19]	
Granulopoiesis	
Neutropenia (common)	Granulocytic hyperplasia
Neutrophilia (uncommon)	Granulocytic hypoplasia
Acquired Pelger–Huët anomaly	Increased blast cells, with or without Auer rods or with giant (pseudo-Chédiak–Higashi) granules
Neutrophils with hypersegmented nuclei, increased nuclear projections, ring nuclei or nuclei of bizarre shape, increased chromatin clumping, detached nuclear fragments‡, increased apoptotic forms	Hypogranular or hypergranular promyelocytes
	Hypogranular myelocytes
	Increased chromatin clumping in myeloid precursors
Agranular and hypogranular neutrophils	Increased monocytes and promonocytes
Hypergranular neutrophils or giant granules (uncommon)	Cytoplasmic vacuolation
Persistence of cytoplasmic basophilia in mature neutrophils or presence of Döhle bodies	Lack of mature neutrophils
	Morphologically abnormal neutrophils
Macropolycytes and binucleated neutrophils	Increased§ or dysplastic eosinophils [20] including eosinophils with intranuclear Charcot–Leyden crystals [21] (uncommon)
Monocytosis, abnormal monocytes	
Presence of promonocytes	
Blast cells, with or without Auer rods	Increased basophils¶ [20]
Eosinophilia (uncommon)	Increased mast cells
Hypogranular eosinophils and eosinophils with ring-shaped nuclei or non-lobulated nuclei	Atypical mast cells [22]
Basophilia (uncommon)	
Thrombopoiesis	
Thrombocytopenia (common)	Reduction of megakaryocytes
Thrombocytosis (uncommon)	Increase of megakaryocytes
Giant platelets	Mononuclear or binuclear micromegakaryocytes
Hypogranular or agranular platelets	Megakaryocytes with hypolobulated nuclei
Platelets with giant granules	Multinucleated megakaryocytes
Micromegakaryocytes	Megakaryocytes with botryoid nuclei
Cyclical thrombocytopenia (rare) [23]	Hypogranular megakaryocytes

* Microcytic anaemia in MDS may be consequent on acquired haemoglobin H disease [24] or, rarely, acquired α [25] or β thalassaemia trait [26]; it can also occur in association with sideroblastic erythropoiesis, although macrocytic anaemia is much more characteristic.

† Elliptocytosis has been linked to an acquired deficiency of protein 4.1 [15] and to del(20q) [16].

‡ However, these are more common in HIV infection and in drug-induced myelodysplasia then in MDS.

§ In one series of patients, bone marrow eosinophils were above 5% in 12.5% of patients [20].

¶ In one series of patients, bone marrow basophils were above 1% in 11.8% of patients [20]

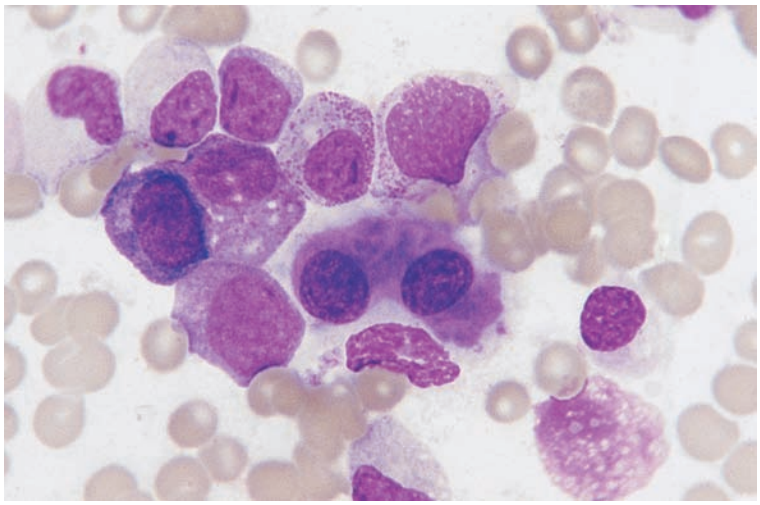


Fig. 4.2 A binucleate micromegakaryocyte in the BM of a patient with refractory cytopenia with multilineage dysplasia (RCMD); there was also granulocytic hyperplasia, hypogranularity of the neutrophil series and severe erythroid hypoplasia. MGG ×100.

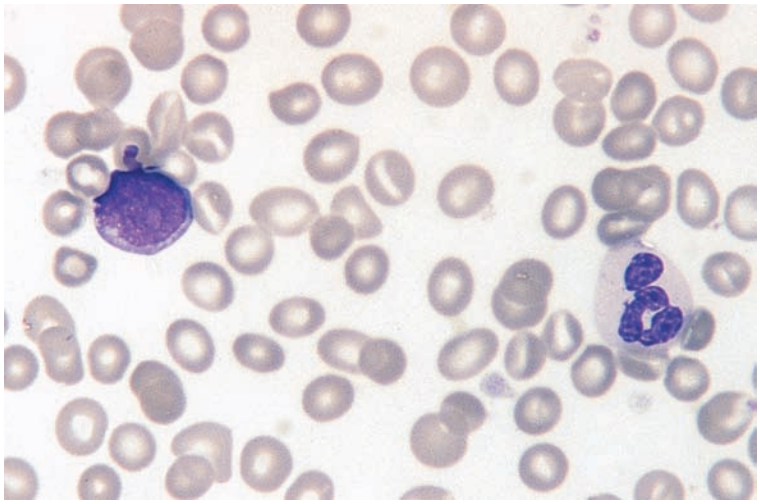


Fig. 4.3 Agranular neutrophil in the PB of a patient with refractory anaemia with excess of blasts (RAEB). A myeloblast is present, some red cells are stomatocytic and platelet numbers are markedly reduced. MGG ×100.

patients. Hypogranular neutrophils are much less specific, partly because of an element of subjectivity in their assessment. Pseudo-Chédiak–Higashi granules probably have a high degree of specificity but are rare (see Fig. 4.27). Other abnormalities such as macrocytosis, red cell fragmentation (Fig. 4.4) or the presence of other poikilocytes, monocytosis, neutropenia, heavy granules in neutrophils or precursors (Fig. 4.5) and binucleate or other apparently tetraploid neutrophils (Fig. 4.6) are less specific and require that alternative diagnoses be excluded. In some patients the features at presentation may not be sufficient to make a firm diagnosis but on continued follow-up the diagnosis becomes certain as disease progression occurs.

The diagnosis of MDS may be aided by ferrokinetic studies to demonstrate ineffective erythropoiesis, cytochemistry (see page 228), immunophenotyping, bone marrow histology (see page 230), bone marrow culture (see page 238) and cytogenetic or molecular genetic analysis to demonstrate acquired clonal abnormalities (see page 234).

Myelodysplastic syndrome needs to be distinguished from the myeloproliferative neoplasms (MPN), among which are included polycythaemia vera, essential thrombocythaemia, primary myelofibrosis

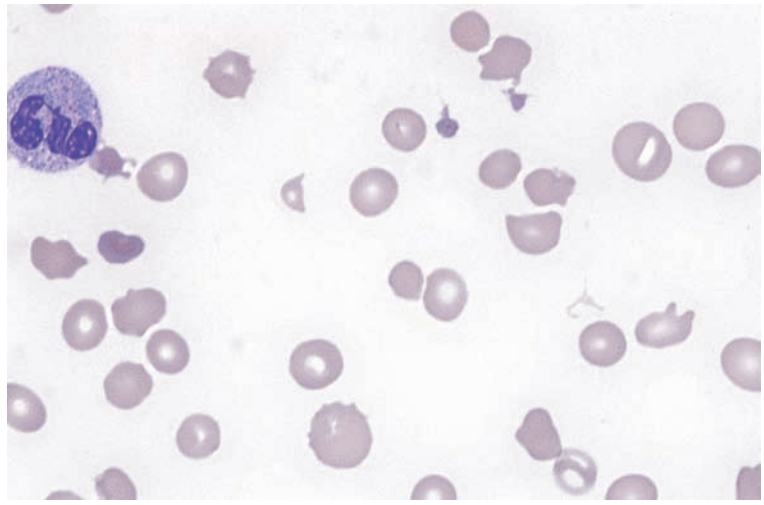


Fig. 4.4 PB film of a patient with RCMD showing marked poikilocytosis including the presence of several fragments and one stomatocyte. There is also anisocytosis with the presence of several macrocytes. Platelet numbers are greatly reduced and BM examination showed megakaryocytic as well as erythroid dysplasia. MGG ×100.

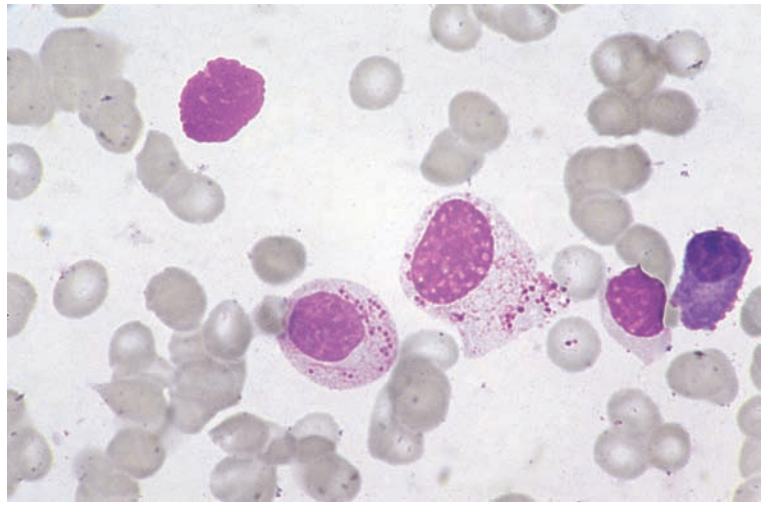


Fig. 4.5 Granulocyte precursors with abnormally heavy granules from a patient with MDS. MGG ×100. (By courtesy of Dr David Swirsky, Leeds.)

and the chronic myeloid leukaemias. In MPN, haemopoiesis is generally effective and excessive leading to features such as erythrocytosis, thrombocytosis, neutrophilia and basophilia whereas in MDS haemopoiesis is generally ineffective with increased cell death in the marrow leading to various cytopenias.

MDS needs also to be separated from a group of conditions that have overlapping features of MDS and MPN. In the WHO classification these constitute a separate group, designated myelodysplastic/myeloproliferative neoplasms (MDS/MPN). Chronic myelomonocytic leukaemia, which was classified as MDS by the FAB group, is now assigned to this new category in acknowledgement of its overlapping features [6,28]. Some patients with refractory anaemia (RA) or refractory anaemia with ring sideroblasts (RARS), as defined by the FAB group, have effective production of platelets so that thrombocytosis rather than thrombocytopenia is seen. If the platelet count is 450×10^9/l or higher such cases are assigned to the WHO MDS/MPN category [28]. MDS, MPN and MDS/MPN can all terminate in acute leukaemia. Myelodysplastic features may appear in patients with MPN and the likelihood of acute leukaemia is then greatly

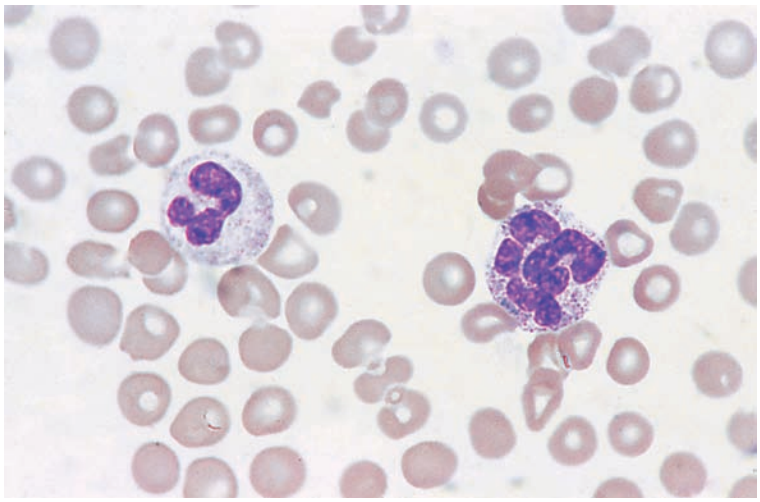


Fig. 4.6 PB film showing a normal neutrophil and a macropolycyte, probably a tetraploid cell, in a patient with MDS. MGG ×100.

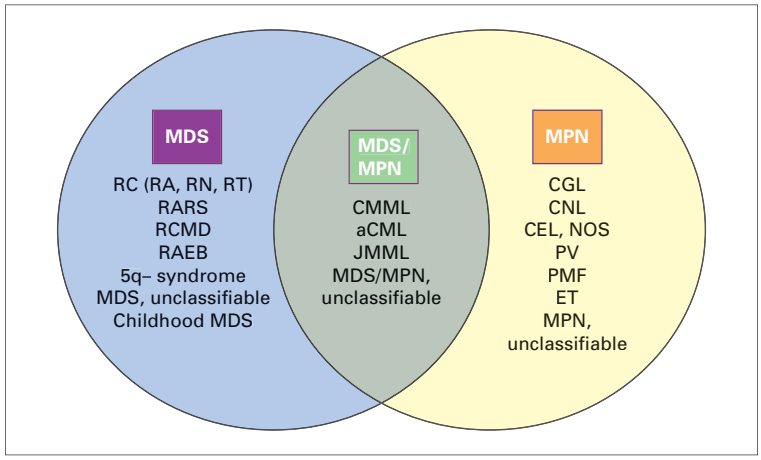


Fig. 4.7 The relationship between the myelodysplastic syndromes, the myeloproliferative neoplasms and the myelodysplastic/myeloproliferative neoplasms in the World Health Organization (WHO) classification. aCML, atypical chronic myeloid leukaemia; CEL, NOS, chronic eosinophilic leukaemia, not otherwise specified; CGL, chronic granulocytic leukaemia; CMML, chronic myelomonocytic leukaemia; CNL, chronic neutrophilic leukaemia; ET, essential thrombocythaemia; JMML, juvenile myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MPN, myeloproliferative neoplasm; PMF, primary myelofibrosis; PV, polycythaemia vera; RA, refractory anaemia; RAEB, refractory anaemia with excess of blasts; RARS, refractory anaemia with ring sideroblasts; RC, refractory cytopenia; RCMD, refractory cytopenia with multilineage dysplasia; RN, refractory neutropenia; RT, refractory thrombocytopenia.

increased. Features such as hypogranular and hypolobulated granulocytes often develop during the course of primary myelofibrosis. Sideroblastic erythropoiesis, the acquired Pelger–Huët anomaly and very small, presumably diploid, megakaryocytes may appear during transformation of chronic granulocytic leukaemia and may herald blast crisis. Similarly, in some patients with polycythaemia vera, a myelodysplastic phase precedes the development of acute leukaemia. The relationship between MDS, MPN and MDS/MPN is shown diagrammatically in Fig. 4.7.

It is likely that the great majority of cases of MDS arise through a mutation in a multipotent stem cell

capable of giving rise to cells of all myeloid lineages. Less often the mutation occurs in a pluripotent stem cell capable of giving rise to lymphoid and myeloid cells. These generalizations are suggested by studies of glucose-6-phosphate dehydrogenase (G6PD) alloenzymes [29], by cytogenetic analysis [30] and by investigation of *RAS* gene mutations and of active and inactive alleles of X chromosome genes [31]. Lineages that appear morphologically normal may be part of the abnormal clone and in some cases the T lymphocytes [30] or both T and B lymphocytes [29,31] are also clonal; lymphocytopenia is common in MDS. The myelodysplastic clone is unstable and with the passage of time clonal evolution occurs, often associated with the acquisition of new karyotypic abnormalities and showing itself in progressive marrow failure, an increase in the number of blast cells or the development of overt acute leukaemia, which is usually myeloid but occasionally lymphoblastic. In rare cases, cytogenetic analysis has proven that the lymphoblasts arise from the myelodysplastic clone [32].

Classification

The FAB classification of MDS was based on morphology of the blood and bone marrow supplemented by a cytochemical stain for iron (Perls' stain). It is summarized in Table 4.2 [4,5] and Fig. 4.8 and median survivals and the likelihood of progression to AML in different categories are shown in Tables 4.3 and 4.4 [33–44]. The FAB classification, which became widely accepted, was crucial in defining the myelodysplastic syndromes, led to their increasing recognition and, by providing a common language through which haematologists could communicate, also led to improved diagnosis and management. It provided, too, a framework for research into the cytogenetic and molecular genetic abnormalities underlying these disorders. However, during the decades following its publication new information, particularly on correlates of prognosis, became available and indicated the need for modification of the classification. The WHO classification was developed using the FAB classification as a basis but incorporating this new knowledge.

There are several quite significant differences between the two classifications. Firstly, whereas the FAB group classified cases as AML rather than MDS if there were 30% or more blast cells in the bone marrow, the WHO classification categorizes cases as AML if there are 20% or more blast cells in either the bone marrow or the peripheral blood. Secondly, cases of CMML are assigned to the MDS/MPN group rather than to MDS. Thirdly, therapy-related MDS,

Table 4.2 The French–American–British (FAB) classification of the myelodysplastic syndromes [4,5].

Category	Peripheral blood		Bone marrow
Refractory anaemia (RA) or refractory cytopenia*	Anaemia,* blasts ≤1%, monocytes ≤1 × 10^9/l	AND	Blasts <5%, ringed sideroblasts ≤15% of erythroblasts
Refractory anaemia with ringed sideroblasts (RARS)	Anaemia, blasts ≤1%, monocytes ≤1 × 10^9/l	AND	Blasts <5%, ringed sideroblasts >15% of erythroblasts
Refractory anaemia with excess of blasts (RAEB)	Anaemia, blasts >1%, monocytes ≤1 × 10^9/l blasts <5%	OR AND	Blasts ≥5% BUT Blasts ≤20%
Chronic myelomonocytic leukaemia (CMML)	Monocytes >1 × 10^9/l, granulocytes often increased, blasts <5%		Blasts up to 20%, promonocytes often increased
Refractory anaemia with excess of blasts in transformation (RAEB-T)	Blasts ≥5%	OR Auer rods in blasts in blood or bone marrow OR	Blasts >20% AND Blasts <30%

* Or in the case of refractory cytopenia either neutropenia or thrombocytopenia.

Table 4.3 Median survival in months in the FAB categories of myelodysplastic syndrome.

Number of cases	RA	RARS	CMML	RAEB	RAEB-T	Reference
101	20	14	4	13	2.5	[33]
141	32	76	22	10.5	5	[34]
109	64	71	8	7	5	[35]
237	50	>60	>60	9	6	[36]
107	23	31	9	8	4	[37]
569	26.5	42	12	8.5	5	[38]
109	91	142	47	19	5	[39]
838	65	58	20	16	10	[40]
226	24	36	12	12.5	12	[41]
816	48	80	28	18	6	[42]
1600	37	50	19	12	5	[43]
213			12			[44]

This table includes only data from series containing more than 100 patients and in which the FAB criteria have been applied with minor or no modifications. Figures have been rounded to the nearest month. See Table 4.2 for abbreviation definitions.

Table 4.4 The percentage of patients transforming to acute leukaemia in the FAB categories of myelodysplastic syndrome.

Number of cases	Length of follow-up	RA	RARS	CMML	RAEB	RAEB-T	Reference
101	'Long term'	16	7	0	38	47	[33]
141	4 months to 16 years	11	5	13	28	55	[34]
109	4–9 years	15	0	32	27	50	[35]
256	5 years	26	16	17	66	60	[36]
569	2–12 years	16	4	49	42	58	[38]
838	3–9 years	27	21	47	76	80	[39]
1600	Long term	10	8	13	28	45	[43]
213	Long term			19			[44]

This figure contains only data from series containing more than 100 patients in which the FAB criteria have been applied with minor or no modifications. See Table 4.2 for abbreviation definitions.

which has a much worse prognosis than *de novo* disease, forms a separate category recognized as being very similar in nature to therapy-related AML.

The WHO classification has a larger number of categories in order to have more homogeneous groups for which prognosis can be inferred with more accuracy. Thus, because prognostic differences were found between FAB-defined RA with and without evident myelodysplastic features in other lineages [45], the WHO classification divides these cases between two groups designated refractory cytopenia (RC) or refractory anaemia (RA) with unilineage dysplasia and refractory cytopenia with multilineage dysplasia (RCMD). Similarly, prognostic differences were found between FAB-defined RARS with and without evident myelodysplastic features in other lineages [46,47] (although not in all reported studies [48]). The former group was generally found to have a higher incidence of clonal cytogenetic abnormality, a greater risk of leukaemic transformation and a shorter survival [46,47]. These cases are therefore also assigned to the RCMD group. A distinct, good prognosis group was identified within the FAB RA and RARS categories, specifically MDS associated with an isolated del(5q) (specifically an interstitial deletion of part of the long arm of chromosome 5); it is now a separate category.

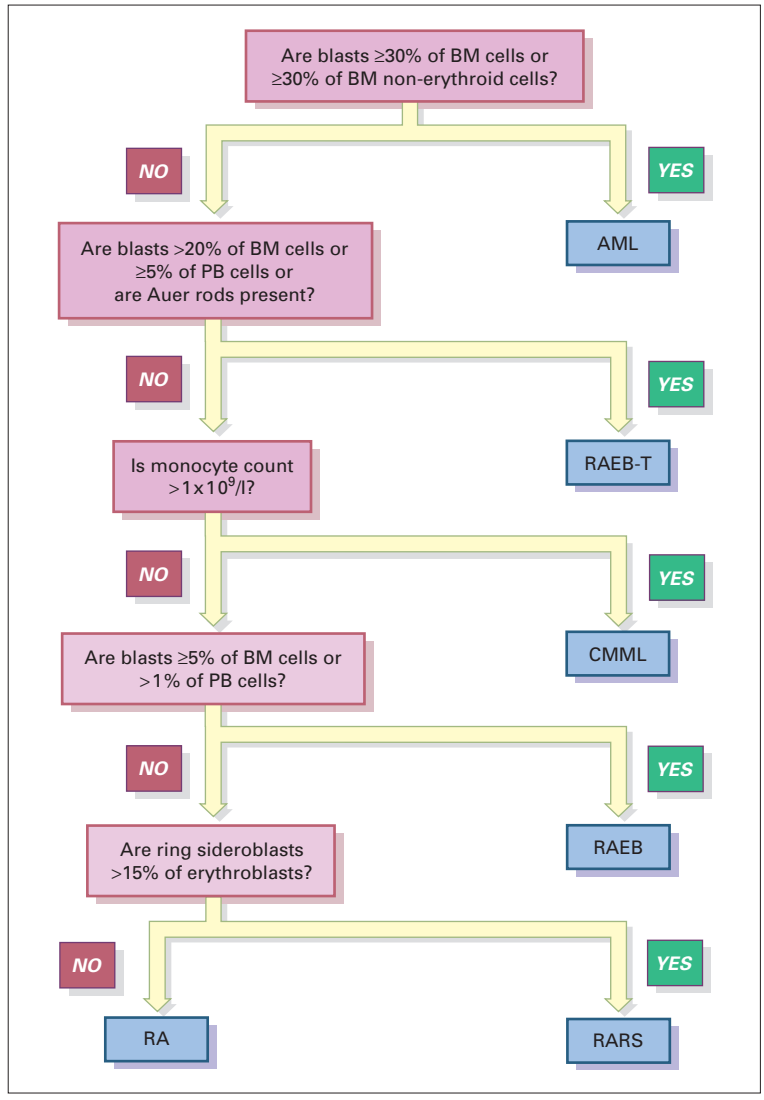


Fig. 4.8 A method of applying the French–American–British (FAB) classification of MDS [4,5]. AML, acute myeloid leukaemia; RAEB-T, refractory anaemia with excess of blasts in transformation (for other abbreviations, see earlier captions).

Patients could be assigned to the FAB category of refractory anaemia with excess of blasts in transformation (RAEB-T) either because the bone marrow blast count was 20% or more (but less than 30%) or because Auer rods were present. Increasingly, haematologists treated patients who had 20–29% bone marrow blast cells in a similar manner to patients with a diagnosis of AML. The lowering of the threshold for categorization of a case as AML reflects this change in clinical practice. However, the optimal categorization of cases with Auer rods remains somewhat problematical. Cases categorized as RAEB-T on the basis of the presence of Auer rods, but with bone marrow blasts being less than 20%, have been found to have a better prognosis than those categorized as RAEB-T on the basis of the blast count in the peripheral blood or bone marrow [49]. In addition, among patients with increased blast cells those who also have Auer rods were found to have a better prognosis than those who did not [50]. The high complete remission rate of patients with RAEB-T and Auer rods who are

treated with combination chemotherapy [49,50] gives support for the view that the presence of Auer rods is indicative of a condition closely related to acute leukaemia and suggests that such patients differ from others categorized as MDS who may have a lower remission rate. In the WHO classification, cases with Auer rods and fewer than 20% bone marrow blasts are categorized as RAEB-2 whereas those with Auer rods and 20–29% blasts have been 'upgraded' to AML. Another group of patients who have similarly been upgraded to AML are those with one of two specific chromosomal rearrangements, either t(8;21)(q22;q22) or inv(16)(p13.1q22)/t(16;16)(p13.1;q22). These patients are now considered to have AML not only when they have between 20% and 29% bone marrow blast cells (FAB RAEB-T) but also when they have even fewer blast cells (mainly FAB RAEB). The reason for this change is that these patients have a high complete remission rate and a good prognosis if treated as AML, and a bone marrow blast percentage of less than 30% (or indeed less than 20%) is not an indication to delay treatment.

When applying the FAB classification, there were some unclassifiable cases; this area of diagnostic uncertainty is acknowledged by a WHO category 'MDS, unclassifiable'.

The WHO classification permits more accurate deductions about prognosis to be made but there is other information of prognostic value that has not been incorporated into the classification. For this reason, the classification should be used in conjunction with a prognostic scoring system such as the International Prognostic Scoring System (IPSS) [42] or the WHO-classification-based Prognostic Scoring Scheme (WPSS) [51] (see below).

It should be noted that both the FAB classification and the subsequent WHO classification require assessment of the number of blast cells in the bone marrow aspirate. There may be a considerable difference between the blast percentage estimated from the aspirate and that estimated from a trephine biopsy section, and the estimation of immature cells from flow cytometry may also differ. In one study the count of CD34-positive or CD117-positive cells was of greater prognostic significance than the blast cell count in the aspirate or the trephine biopsy specimen [52]. Nevertheless, all classifications and prognostic scoring systems are based on the number of blast cells counted in blood and bone marrow films.

The updated WHO classification [7] is now widely used and has largely replaced the FAB classification; the FAB classification will therefore not be discussed in detail. Readers who require further information are referred to the third edition of this book [53] and to the original papers of the FAB group [3–5].

Cytochemistry

The only cytochemical reactions essential in the diagnosis and classification of MDS are a Perls' stain for iron, which is necessary for assessing the presence and number of ring sideroblasts, and a Sudan black B (SBB) or myeloperoxidase (MPO) stain to ensure that all cases with Auer rods are recognized. These and other cytochemical reactions may provide evidence of dysplastic maturation and can thus be useful both in confirming the diagnosis and in assessing the number of lineages involved.

Cytochemical stains for MPO, SBB and naphthol AS-D chloroacetate esterase (chloroacetate esterase, CAE) may show mature neutrophils with negative reactions [54] and, similarly, mature monocytes may be deficient in non-specific esterase (NSE) activity. MPO activity and the MPO antigen are often reduced in a similar proportion of neutrophils but sometimes reduction in activity is seen in a significantly greater proportion of neutrophils, indicating a protein with defective enzymatic activity [55]. The neutrophil alkaline phosphatase score is reduced in a third to a half of patients and in a minority is elevated. Immunocytochemistry can similarly show lack of elastase (a primary granule constituent) and lactoferrin (a secondary granule constituent) in a significant proportion of neutrophils in many patients with MDS [55].

When patients with MDS have an increase of bone marrow blasts the cells show the cytochemical reactions expected of myeloblasts, monoblasts or megakaryoblasts. Relatively undifferentiated myeloblasts are the form most commonly present so that positive reactions for SBB, MPO and CAE may be weak and confined to a minority of cells. In some cases the blasts do not give positive reactions with any cytochemical markers of myeloid cells. Such cells are generally very immature myeloblasts,

confirmed by immunophenotyping, but in a minority of cases the cells appear to be lymphoblasts.

Erythroblasts may be periodic acid–Schiff (PAS) stain positive with the positive reaction being confined to proerythroblasts or being present in early, intermediate and late erythroblasts [56]. Although PAS positivity is seen in some reactive conditions its presence in patients with MDS or leukaemia is likely to indicate that the lineage is part of the abnormal clone. Erythroblasts may also show aberrant, strong, paranuclear positivity for acid phosphatase and NSE; such reactions are not confined to MDS and AML but may be seen in megaloblastic anaemia [54]. In MDS, the percentage of haemoglobin F and the percentage of cells containing haemoglobin F may both be increased. A cytochemical stain such as the Kleihauer reaction will identify the increased percentage of cells containing haemoglobin F. Such an increase is not confined to MDS and in fact is most characteristic of juvenile myelomonocytic leukaemia (see Chapter 5). A minority of cases of MDS have acquired haemoglobin H disease [24]; they can be identified with a cytochemical stain using new methylene blue or brilliant cresyl blue (as well as by haemoglobin electrophoresis).

Immunophenotyping

Immunophenotyping has generally been considered to have only a limited place in the diagnosis of MDS. It has a role in demonstrating or confirming the nature of blasts, particularly when myeloblasts are very immature and lack characteristic cytochemical reactions, when megakaryoblasts are present or, uncommonly, when there is a lymphoblastic or mixed phenotype transformation of MDS. It can be applied to bone marrow films to identify dysplastic megakaryocytes including micromegakaryocytes (Fig. 4.9).

However, if multicolour flow cytometry with an extensive panel of reagents is used, in conjunction with assessment of light-scattering characteristics of cells, immunophenotyping can be more informative and can give strong support for a diagnosis of MDS or MDS/MPN in patients lacking convincing morphological evidence of this diagnosis [57–61]. Abnormalities demonstrated in the granulocytic lineage may include detection of hypogranular neutrophils (reduced sideways light scatter), a population of cells in the 'blast window' (identified on a CD45/sideways light scatter plot), abnormal antigen expression by blast cells (e.g. reduced CD45 expression; lack of expression of human leucocyte antigen DR (HLA-DR), CD13 or CD33; inappropriate expression of CD5, CD7, CD11b, CD16, CD19 or CD56) and detection of abnormal antigen expression by maturing cells, either granulocytes or monocytes (reduced expression of CD11b, CD16 or CD33 or expression of CD34 or non-myeloid antigens such as CD5, CD7 or CD56) [57,58,61,62]. Abnormalities detected in erythroid cells include

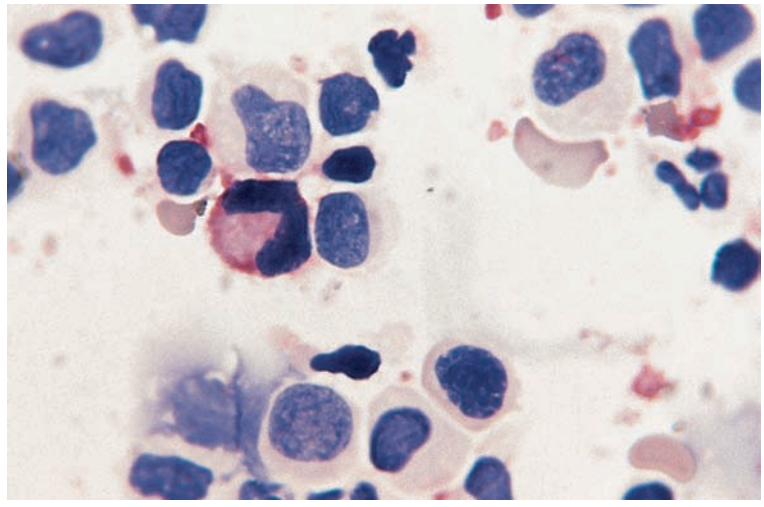


Fig. 4.9 Immunocytochemistry of a bone marrow film from a patient with RAEB-T in the FAB classification, classified as acute myeloid leukaemia in the WHO classification, showing several platelets and a micromegakaryocyte, which have given positive reactions with a monoclonal antibody directed at platelet glycoprotein Ib (CD42b). Alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique ×100.

reduced CD71 expression and asynchronous expression of CD71 or glycophorin A in comparison with CD45 [57]. It is possible to use the number of immunophenotypic abnormalities to assess the degree of dysplasia; a scoring system has been devised that is useful for prognosis as well as for supporting a diagnosis [58]. Another scoring system uses a small number of antibodies – CD66 (increased), CD11a (increased), CD10 (occasionally decreased) and CD116 (occasionally either increased or decreased) – together with sideways light scatter (reduced) [62]. Expression of CD7 and CD56 on myeloid blasts has been found to be an independent adverse prognostic indicator [63].

It is necessary to note that immunophenotypic analysis gives evidence of abnormal maturation, rather than specifically of MDS. Abnormalities can be seen in a regenerating marrow (aberrant CD56 expression) [61], in sepsis (aberrant expression of CD64 on neutrophils) [61], in systemic lupus erythematosus (reduced monocyte expression of CD14 and HLA-DR) [64] and no doubt in other non-neoplastic conditions characterized by dysplasia.

There appears to be little relationship between immunophenotype and FAB or WHO categories of MDS.

Immunophenotyping can also be carried out by immunohistochemistry in trephine biopsy sections (see below).

Bone marrow trephine biopsy

Bone marrow aspiration and trephine biopsy are complementary investigations. A core biopsy often gives extra information not provided by an aspirate [65–69]. Cellularity can be more reliably assessed and increased reticulin is apparent. Cellularity is most often increased but may be normal or decreased. Abnormal distribution of cells is often detectable. Erythroid islands may be absent or very large (Fig. 4.10). They may show an excess of proerythroblasts (Fig. 4.11) or have all precursors at the same stage of development [65]. Late in the disease course there may be marked reduction of erythropoiesis. Granulocytic precursors may be clustered centrally rather than showing their normal paratrabecular distribution (Figs 4.12 and 4.13). This phenomenon has been designated 'abnormal localization of immature precursors' (ALIP) [65]. ALIPs can be diagnostically important if they are detected in RA since their presence confirms MDS rather than a secondary anaemia. Abnormal megakaryocytes (mononuclear, binuclear and multinucleated forms) are readily assessed on a biopsy (Fig. 4.14). Megakaryocytes may be clustered or found in a paratrabecular position (Fig. 4.15). Dysgranulopoiesis and dyserythropoiesis may be detectable but they are more readily apparent in a bone marrow film. Apoptosis is increased [70]. Nodules of immunophenotypically abnormal

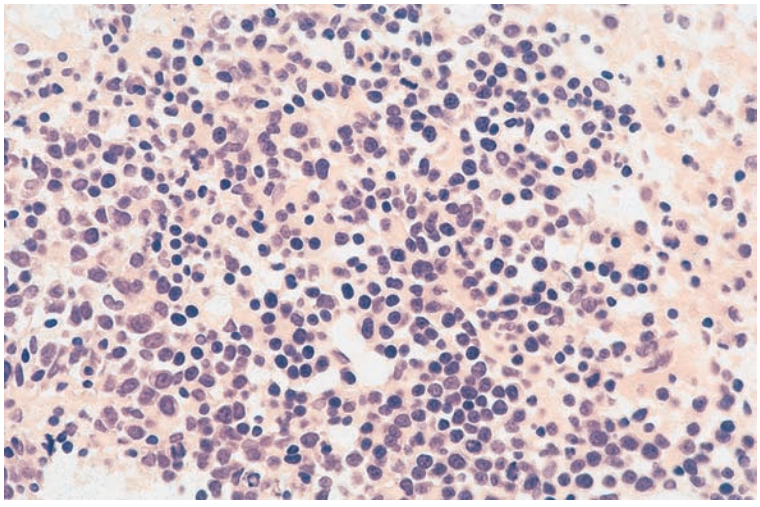
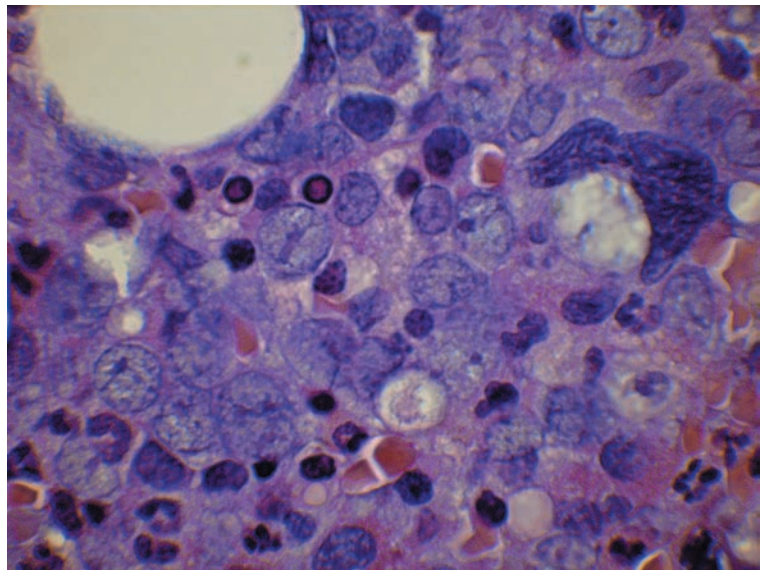
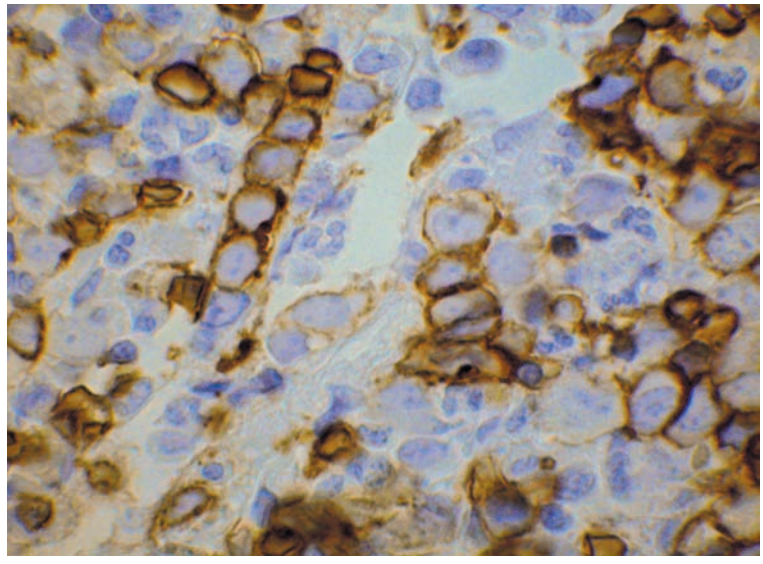


Fig. 4.10 Histological section of a trephine biopsy specimen from a patient with RAEB showing a large ill-formed erythroid island. Haematoxylin and eosin (H&E) ×40.



(a)



(b)

Fig. 4.11 Histological section of a trephine biopsy specimen from a patient with MDS showing very numerous proerythroblasts (note the typical elongated nucleoli in several of the proerythroblasts: (a) H&E ×100; (b) immunoperoxidase for glycophorin ×100;

Continued

monocytes (or plasmacytoid dendritic cells) are sometime present [71]. Biopsy sections may show non-specific abnormalities such as increased macrophages, prominent mast cells, lymphoid follicles and plasma cell aggregates. Ring sideroblasts are usually detectable only in sections of resin-embedded specimens. Sections of paraffin-embedded trephine biopsies not only do not permit the detection of ring sideroblasts but also do not permit a reliable assessment of iron overload since iron may be leached out during decalcification.

Immunohistochemistry is an important supplement to histology. Anti-glycophorin antibodies highlight the presence of clusters of immature erythroid cells and help to distinguish them from ALIPs. Polyclonal antibodies directed at von Willebrand factor or monoclonal antibodies recognizing epitopes on platelet antigens, such as CD42a, CD42b

(c)

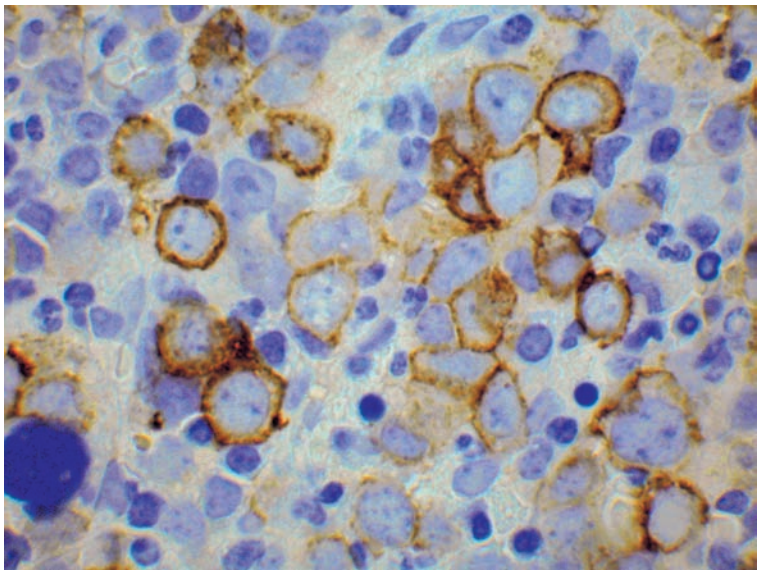


Fig. 4.11 (*Continued*) (c) immunoperoxidase for CD117 (which can be expressed by proerythroblasts as well as by early cells of granulocyte lineage) ×100. (By courtesy of Dr Alan Mills, Bendigo.)

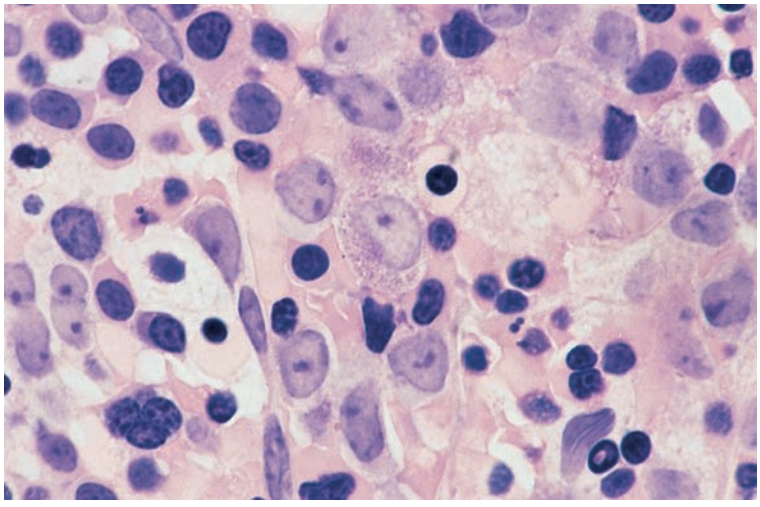


Fig. 4.12 Histological section of a trephine biopsy specimen from a patient with RAEB-T showing erythroblasts, some of which are dysplastic, and a collection of blasts and promyelocytes that are not adjacent to bone – 'abnormal localization of immature precursors' (ALIP). H&E ×100.

or CD61, highlight megakaryocytes and facilitate the recognition of small mononuclear megakaryocytes. Expression of granulocyte antigens can confirm the nature of ALIPs (see Fig. 4.13). Monocyte/plasmacytoid dendritic cell nodules are strongly positive for CD68 but they may fail to express lysozyme; in about half of cases there is expression of CD123 [71]. Reactions with some antibodies give prognostic information [72]. Increased numbers of CD34-positive cells and positive reactions for the p53 protein (a protein encoded by a tumour-suppressor gene, *TP53*) are mainly seen in RAEB and are indicative of a worse prognosis. p53 positivity is usually indicative of the fact that there is a mutated gene present, the product of which has a longer half life than the normal protein; this interpretation is confirmed by the absence of an equivalent overexpression of p21.

The experience of different groups differed as to whether FAB subtypes of MDS constituted recognizable histological entities. Tricot *et al.* [65] could not recognize entities that corresponded to

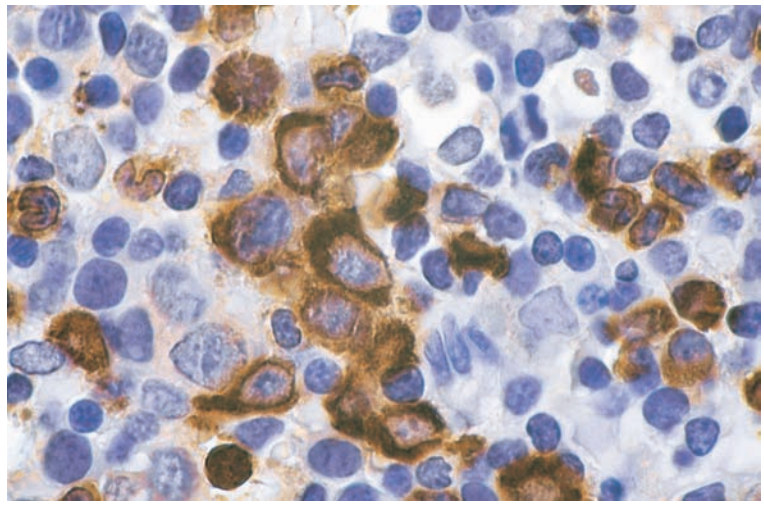


Fig. 4.13 Histological section of a trephine biopsy specimen from a patient with RARS showing an ALIP demonstrated by immunohistochemistry. Immunoperoxidase ×100.

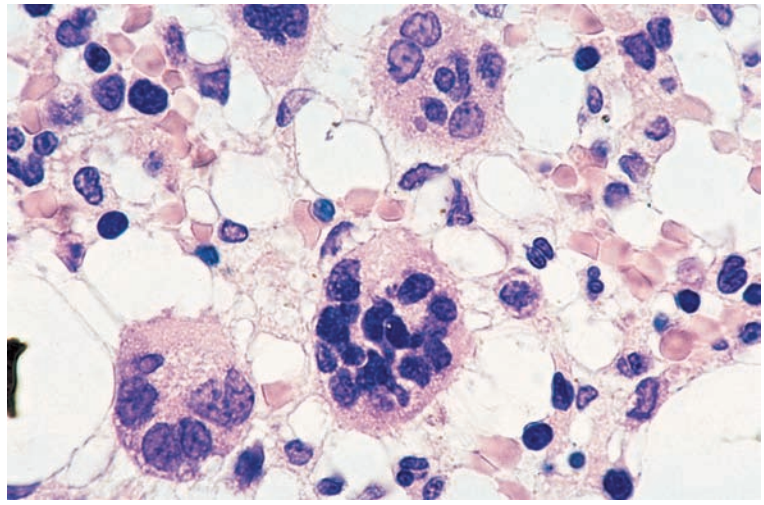


Fig. 4.14 Histological section of a trephine biopsy specimen from a patient with RAEB showing dysplastic megakaryocytes and general disorganization of BM architecture. H&E ×100.

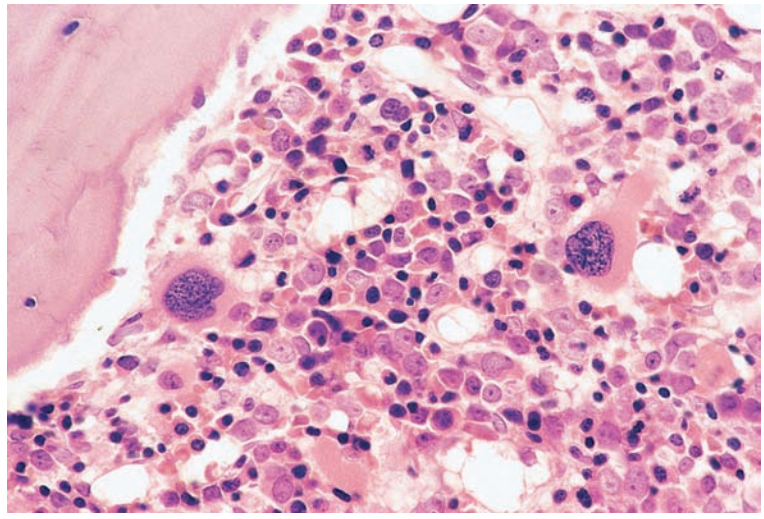


Fig. 4.15 Histological section of a trephine biopsy specimen from a patient with MDS showing abnormal megakaryocyte topography; a megakaryocyte is abnormally sited, adjacent to the bony trabecula. H&E ×40. (By courtesy of Dr Simon Davies.)

the FAB categories. Delacrétaz *et al.* [67], however, reached concordant diagnoses in 24 of 28 cases examined independently. Both groups found ALIP in more than half the biopsies but Tricot *et al.* [66] found them in all subtypes of MDS (although preferentially in FAB RAEB and RAEB-T) whereas Delacrétaz *et al.* [67] found ALIP to be very uncommon in cases that did not have an excess of blasts in the aspirate. Identification of specific WHO subtypes from trephine biopsy sections is likely to be even more unreliable since diagnostic criteria depend on precise quantification of cell types and cytological abnormalities.

A core biopsy is particularly useful in assessing cases with a normocellular or hypocellular bone marrow and those cases with increased reticulin, in which a poor aspirate that is unlikely to be representative is obtained. It is thus particularly likely to be useful in therapy-related MDS (t-MDS) in which both reduced cellularity and increased reticulin are much more common than in *de novo* cases. A biopsy is helpful in distinguishing hypocellular MDS, which may have increased reticulin and foci of blasts, from aplastic anaemia, which does not show these features.

Cytogenetic and molecular genetic analysis

Various clonal cytogenetic abnormalities have been described in association with MDS [73–82] (Table 4.5) among which the commonest anomalies are del(5q) (often referred to as 5q–), monosomy 5, del(7q) (also referred to as 7q–), monosomy 7 (Fig. 4.16) and trisomy 8. Some abnormalities are particularly characteristic of t-MDS (Table 4.5). MDS is commonly associated with loss of chromosomal material, either through monosomy, deletion or unbalanced translocation. Balanced translocations are less common.

There is no close relationship between specific cytogenetic abnormalities and FAB categories, although some trends are apparent. The frequency with which cytogenetic abnormalities are observed increases from 30–50% in FAB-defined RA and RARS to about 60% in RAEB and almost 70% in RAEB-T [79]. Single abnormalities are most likely to be found in RA and RARS whereas RAEB-T shows the highest rate of occurrence of complex rearrangements (whether this is defined as a minimum of two, three or four abnormalities in a karyotype). The commonest abnormalities, –5, del(5q), –7, del(7q) and +8, are seen in all FAB categories but del(5q) as a single defect is preferentially associated with good prognosis subtypes and with the features of the '5q– syndrome' (see below). Abnormalities associated with RA are +8, del(20q), –7, i(17q) and del(17p). The uncommon chromosomal rearrangement, idic(X)(q13), is associated with RARS [77]. In some series of patients rearrangements of chromosome 11 have been associated preferentially with RARS.

The WHO classification recognizes the '5q– syndrome' as an entity (see below). Otherwise the relationship of the WHO categories to cytogenetic

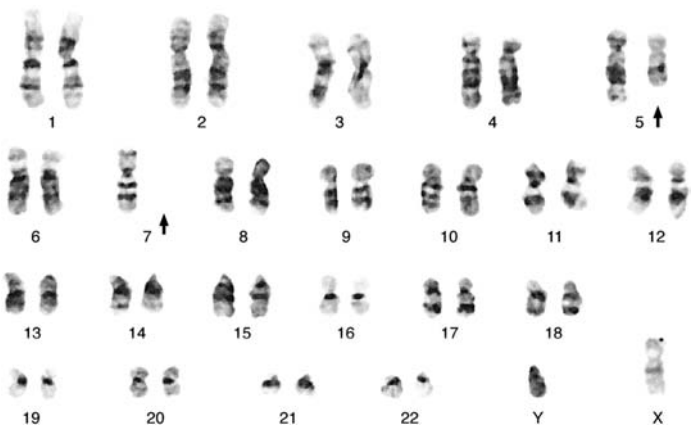


Fig. 4.16 Karyogram of a male patient with MDS showing a partial deletion of the long arm of chromosome 5 and monosomy 7. The karyotype was 45,XY,del(5)(q21q33),–7. (By courtesy of Professor Lorna Secker-Walker.)

Table 4.5 Cytogenetic abnormalities associated with the myelodysplastic syndromes. (Derived from references 42,73–82, and other sources.)

	Loss of chromosomal material	Gain of chromosomal material	Chromosomal rearrangement
Common	–5* del(5q) –7* del(7q)* del(9q) del(20q)* –Y*†	+8	
Less common	del(1p) del(11q) del(12p)* del(13q) del(17p)* –17* –20 del(21q) –22		11q23 rearrangements including: t(2;11)(p21;q23) t(9;11)(p21;q23)* t(11;16)(q23;p13.3)* del(11)(q23) i(17q) 21q22 rearrangements including: t(3;21)(q26.2;q22)*
Uncommon or rare rearrangements	del(3p)* del(6p)* –8 –14 del(14q) –15 del(16q) del(17q)* del(18q) –18* –19 –21	+4 +6 +11 +13 +14 +16 +19 +21	Xp11* and Xp13* rearrangements including: idic(X)(13) 3q21 and/or 3q26 rearrangements: t(1;3)(p26;q21)* inv(3)(q21.1q26.2)* t(3;3)(q21.1;q26.2)* del(3)(q21) ins(3)(q26;q21q26) t(3;4)(q26;q21) t(3;5)(q21;q31)* t(3;8)(q26;q24) t(3;12)(q26;p13) t(3;19)(q21;p13) t(3;5)(q25;q34) t(6;9)(p23;q34.5) 11q23 rearrangements less often associated with MDS including: t(1;11)(p32;q23)* t(3;11)(p21;q23) t(11;17)(q23;q25) t(11;19)(q23;q13.1)* t(11;21)(q24;q11.2) Rearrangements of 17q21* Rearrangements of 19p13* or 19q13* –20, ider(20q)†† Ring chromosomes Double minute chromosomes

Chromosomal rearrangements that are usually unbalanced:
- der(Y)t(Y;1)(q12;q21)¶
- der(1)t(1;7)(q10;p10)*§¶
- der(1)t(1;13)(q11;q10)¶
- der(1)t(1;15)(q12;p11)¶
- der(16)t(1;16)(q11;q11)*¶
- –5,–7,+der(5)t(5;7)(q11;p11)*
- –17,dic(5;17)(p11-13;p11-13)**
- –17,t(7;17)(p11;p11)**
- –12,der(17)t(12;17)(q13;p13)*

* Commoner among cases of t-MDS.
† –Y is usually an age-related change rather than being indicative of clonal haemopoiesis [74].
§ This translocation has also been described as dic(1;7)(p11;q11) and as t(1;7)(cen;cen).
¶ These translocations result in trisomy for all or part of 1q.
** These translocations result in deletion of 17p.
†† There is both gain and loss of chromosomal material.

abnormalities is similar to that described for the FAB categories, with clonal abnormalities and, in particular, complex karyotypic abnormalities being more common in the categories of MDS with a worse prognosis. Not surprisingly, adverse cytogenetic abnormalities are found to be more common in the WHO RCMD category than in RA and RARS [83,84].

Certain abnormalities are associated with particular clinical or morphological features but not with specific FAB or WHO categories. Monosomy 7 is seen in all FAB categories but is more often found when there is pancytopenia, a hypocellular bone marrow, trilineage myelodysplasia and a relatively poor prognosis. Hypocellular MDS has shown an association with trisomy 6, trisomy 8, del(7q) and del(5q). Del(11q) has been associated with increased ring sideroblasts whether or not the disease falls into the RARS category. Marked elliptocytosis has been associated with del(20q), this cytogenetic abnormality being observed in 6 of 9 studied cases [16]. Isolated del(20q) is also significantly associated with lower blast cell counts [85] and thrombocytopenia [85,86].

Abnormalities of 3q21 and 3q26 are associated with thrombocytosis and with increased and abnormal megakaryocytes; the megakaryocytes are more pleomorphic than those of the 5q– syndrome. Dysplastic megakaryocytes may include micromegakaryocytes, multinucleated megakaryocytes and large non-lobulated forms. Some patients with 3q21q26 abnormalities will meet the diagnostic criteria for MDS while others have MDS/MPN or AML.

Deletion of the short arm of chromosome 17, with formation of an isochromosome of the long arm, i(17q) (Fig. 4.17), has been found to be associated with MDS and MDS/MPN with distinctive haematological features and a poor prognosis; this has sometimes been referred to as the 17p– syndrome. Cases are usually FAB categories RA, RAEB, RAEB-T or CMML. A significant proportion are therapy related. There is moderate to severe anaemia and characteristic dysgranulopoiesis with an acquired Pelger–Huët anomaly of neutrophils and eosinophils, small vacuolated neutrophils and MPO deficiency [87–89]. The neutrophils may also be hypogranular and they may have ring nuclei [89]. There is usually trilineage myelodysplasia (including micromegakaryocytes [89]) and although the blast count is often less than 5% at presentation there is rapid progression to AML [90]. There may be myeloproliferative as well as myelodysplastic features with most patients having leucocytosis and monocytosis and some having thrombocytosis [90], this leading to categorization of these cases, in the WHO classification, as MDS/MPN. Immature granulocytes may be increased in the peripheral blood, some patients have eosinophilia and a smaller number have basophilia [90]. A trephine biopsy shows increased reticulin fibrosis [90]. Del(17p) can be detected by interphase fluorescence *in situ* hybridization (FISH) as well as by classical cytogenetic analysis. Similar morphological abnormalities occur in other diseases with loss of 17p including

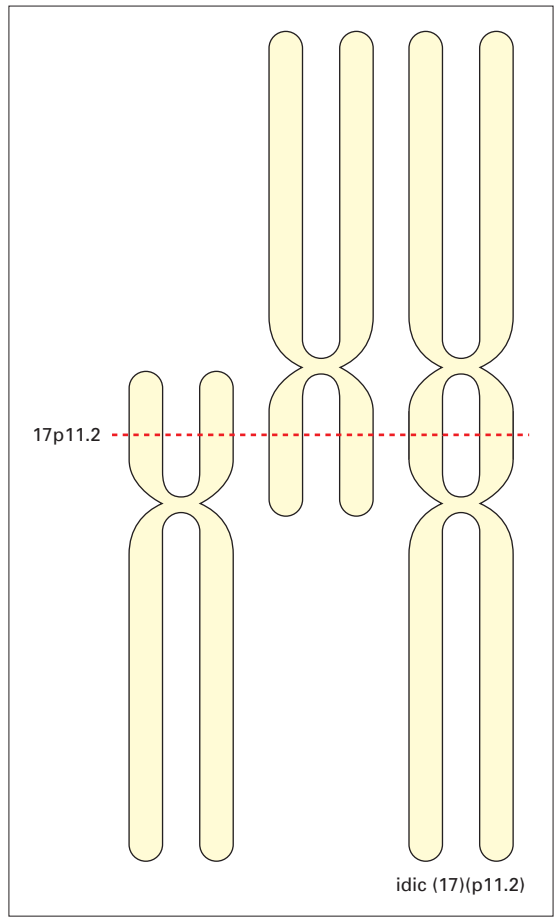


Fig. 4.17 Diagram showing the development of idic(17)(p11.2), the commonest form of i(17q).

AML and MPN in evolution. The 17p– syndrome can result not only from simple deletion of the short arm of 17 but also from monosomy 17, i(17q) and unbalanced translocations with the loss of 17p such as –17,t(5;17)(p11;p11) and –17,t(7;17)(p11;p11). The relevant abnormality is often part of a complex cytogenetic abnormality. The 17p– syndrome is related to loss of the *TP53* tumour-suppressor gene. The other *TP53* allele is often mutated.

In the WHO classification, the presence of specified clonal chromosomal abnormalities can provide the basis for a diagnosis of MDS when cytological abnormalities are not in themselves sufficient for diagnosis (see pages 252–253).

The detection of certain cytogenetic abnormalities is relevant to therapy. A response to lenalidomide is likely in patients with del(5q), whether or not other cytogenetic abnormalities are present and whether or not the case meets the criteria for the 5q– syndrome. A response to azacitidine correlates with the presence of monosomy 7 [91] but is not confined to such cases.

The molecular basis of MDS has been defined only to a limited extent. Characteristically there are multiple genetic lesions, particularly in higher grade MDS. Patients with translocations that are also seen in AML have the same molecular genetic changes as are seen in patients with overt leukaemia. This is true of t(6;9)(p23;q23), abnormalities of 3q21 and 3q26.2 and translocations with 11q23 and 21q22 breakpoints. In patients whose myeloid cells show loss of all or part of a chromosome, e.g. –5, del(5q), –7, del(7q), del(17p) and del(20q), it is possible that deletion of a tumour-suppressing gene is critical in the development of MDS. This has been shown for del(17p) in which deletion of one allele of the tumour suppressor gene, *TP53*, is often accompanied by mutation or submicroscopic deletion of the other allele [92]. Haploinsufficiency for a gene in a commonly deleted region provides an alternative and possibly more common mechanism, which could explain why many years of investigation have not been very fruitful in finding a critical tumour suppressor gene that is consistently deleted for the majority of these abnormalities. Mutations in *RAS* genes (mainly *NRAS*) and, to a lesser extent in *FMS*, are common in MDS (20–40% and 12–20%, respectively) and, like *TP53* mutations, are indicative of a worse prognosis [93]. *RAS* gene mutations may be induced by irradiation. Mutation or deletion of the tumour suppressor gene *TET2* is found in about a fifth of patients with MDS and may be homozygous [94]. *RUNX1* mutations are common, being found in 10% of patients in one study (percentage corrected to exclude RAEB-T and CMML) [95]. *RUNX1* mutations can be induced by both irradiation and anti-cancer chemotherapy but they also occur in *de novo* MDS [95,96]; in *de novo* disease they are associated with higher grade MDS and worse prognosis [95]. *FLT3* mutations (mainly internal tandem duplications) occur in a minority of patients, correlate with trilineage dysplasia and are predictive of transformation to AML [97]. In addition to gene mutation, epigenetic effects may have a role in the development of MDS. Methylation of the promoter of the $p15^{INK4b}$ cyclin-dependent kinase inhibitor gene (*CDKN2B*) is seen in 40–50% of patients, particularly in those with worse prognosis subtypes of MDS. Other genes (e.g. *KIT*, *MDR1* and *MDM2*) are also sometimes overexpressed in the absence of mutation.

Myelodysplastic syndrome may be associated with mutations of mitochondrial as well as nuclear deoxyribonucleic acid (DNA) [98,99]. For example, mutations of cytochrome c oxidase I and II and cytochrome b genes have been described in at least 36 patients and other patients have had mutations in mitochondrial genes encoding transfer ribonucleic acid (mtRNA) [99,100]. However, mitochondrial mutations do not appear to be common [101]. Mitochondrial mutations are likely to precede clonal expansion rather than be the event leading to it [99]. They may sometimes be the cause of sideroblastic erythropoiesis. One reported patient with RARS who had a cytochrome c oxidase mutation developed del(5q) during the course of the disease, illustrating further clonal evolution [83].

Other laboratory tests

Automated blood cell counts

Automated blood cell counters using peroxidase cytochemistry (e.g. the Siemens Advia 120) can give diagnostically useful information in patients with MDS [102] (Fig. 4.18). Peroxidase deficiency of neutrophils can be detected by an abnormal position of the neutrophil cluster; less often

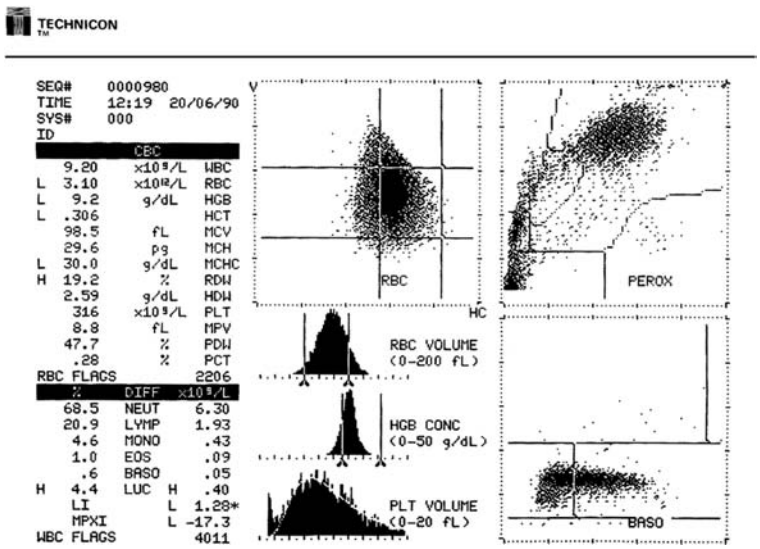


Fig. 4.18 Histograms and scatterplot produced by a Bayer H.1 automated counter in a case of RARS showing both macrocytes and hypochromic cells.

peroxidase-deficient neutrophils are present as a distinct population. In other cases the peroxidase activity of dysplastic neutrophils is increased. The presence of blasts in RAEB can be suspected because of the presence of large peroxidase-negative cells or because of the presence of cells that have nuclei with abnormal light-scattering qualities. Red cell histograms and scattergrams may show increased heterogeneity of red cell size and haemoglobinization, macrocytosis, microcytosis (rarely) or, when there is sideroblastic erythropoiesis, a bimodal distribution of red cell size or haemoglobin concentration. Two overlapping populations can be observed on scatterplots, one of these being a cluster of hypochromic microcytic cells. The platelet histogram may show the presence of giant platelets.

Other automated instruments that size cells by electrical conductivity or light scatter give similar but not identical information about red cell and platelet size distribution but more limited information about white cell characteristics. Reduced mean neutrophil conductivity and scatter on Beckmann–Coulter instruments are found in MDS and reduced scatter has been found to correlate with neutrophil hypogranularity [103]. It should, however, be noted that reduced conductivity and scatter have also been reported in transient dysplasia following chemotherapy [103].

In one study low mean platelet volume was associated with a worse prognosis, as was low 'platelet mass' (the product of the mean platelet volume and the platelet count) [104]. An independent study confirmed the adverse prognostic significance of a low platelet mass but not that of a low mean platelet volume [105].

All automated instruments are able to detect the presence of blast cells in the majority of cases in which they are detected by microscopy.

Platelet function tests

Platelet function is often abnormal, particularly in higher grade MDS [106]; defective platelet function can lead to a bleeding tendency, even in patients with a normal platelet count.

Biochemical tests

Serum ferritin, serum iron and transferrin saturation may be elevated. Higher ferritin levels correlate with a worse prognosis in some but not all studies [107] and in one retrospective study iron chelation therapy correlated with longer survival [108]. The ferritin should therefore be monitored in good prognosis MDS so that iron overload can be detected and managed appropriately.

Lactate dehydrogenase may be elevated in high grade MDS.

Bone marrow culture

In vitro culture of bone marrow cells may show abnormal growth of granulocyte/monocyte progenitors

but this is largely a research tool rather than being used in routine diagnosis. The abnormal pattern may be: (i) reduced colonies; (ii) increased colonies and/or clusters; or (iii) reduced colonies and increased clusters (in some but not all studies predictive of transformation to acute leukaemia). A normal growth pattern of granulocyte–macrophage colony-forming units (CFU-GM) may be seen in low grade MDS (FAB RA, RARS) whereas an abnormal pattern is usual in high grade MDS (FAB RAEB). In one study a marked abnormality of colony growth was observed in RARS with dysplasia of other lineages (i.e. RCMD) but not in RARS without other dysplasia [109]. Growth of BFU-E (erythroid burst-forming units), CFU-E (erythroid colony-forming units) and CFU-Meg (megakaryocyte colony-forming units) is often reduced or absent.

Disease evolution and prognosis

Patients with MDS may die of marrow failure or following transformation to acute leukaemia. The likelihood of either outcome and the rapidity with which such disease evolution occurs vary between the different FAB and WHO categories. Myelodysplastic syndromes may also evolve into other categories of MDS. Change is usually into a worse prognostic category and very rarely into a more favourable category. Thus, RA, RARS and RCMD may evolve into RAEB. When acute leukaemia supervenes it may develop within a brief period or there may be a stepwise evolution over many weeks or months. The acute leukaemia that occurs in MDS is almost always AML but rare cases of acute lymphoblastic leukaemia (ALL) and of mixed phenotype acute leukaemia have been reported; this occurrence is consistent with the evidence suggesting that in at least some cases the cell giving rise to the MDS clone is a pluripotent lymphoid–myeloid stem cell. However, it should be noted that in the great majority of cases of apparent lymphoid or mixed phenotype acute leukaemia it has not been demonstrated that the acute leukaemia has arisen from the MDS clone. Since MDS is predominantly a disease of the elderly a significant proportion of patients with MDS die of other diseases. The likelihood of this outcome is of course greatest in those in the best prognostic categories, RA, RARS and the 5q– syndrome. Occasional patients with these good prognosis types of MDS die of iron overload.

A number of factors can be correlated with prognosis of MDS [34,38,42,43,68,84,110–121] (Table 4.6). The FAB classification divides patients into two broad prognostic groups, RA plus RARS and RAEB plus RAEB-T. There is no consistent or statistically significant difference between median survivals in RA and RARS, although leukaemic transformation is less common in RARS [112] (see Tables 4.3 and 4.4). The prognosis of RAEB-T is somewhat worse than that of RAEB and in one large series of patients the difference in survival was significant [112]. The WHO classification can be used to divide MDS into four prognostic groups (see Table 4.8).

Because of the heterogeneity within MDS categories, efforts have been made to use other criteria to give a clearer idea of prognosis in the individual patient. Cytogenetic analysis is of considerable importance since specific karyotypic abnormalities have independent prognostic significance [39,42,76,84,118,122]. In general, cases with a normal karyotype have been found to have a better prognosis than those with a clonal cytogenetic abnormality, and in some series of patients those with a mixture of normal and abnormal metaphases had a better prognosis than those with only abnormal metaphases. In general, the best prognosis is associated with a normal karyotype, isolated del(5q), isolated del(20q) and isolated –Y (the latter not necessarily a clonal marker). An intermediate prognosis is found in association with trisomy 8 and miscellaneous single and some double defects. The worst prognosis is associated with abnormalities of chromosome 7, complex karyotypes (e.g. defined as three or more unrelated abnormalities) and certain specific translocations: t(1;3)(p36;q21), t(6;9)(p23;q34), rearrangements with an 11q23 breakpoint, 3q21q26 abnormalities and perhaps also t(1;7)(p11;q11) have been associated with a high probability of transformation to AML and poor prognosis. Rearrangements of 11q23 (*MLL* rearranged) are specifically associated with evolution to AML of M4 and M5 categories [82].

Karyotypic abnormalities have been incorporated, together with other variables shown to indicate prognosis, into a number of scoring systems. These include the Lille scoring system [115,122],

Table 4.6 Factors that have been reported to have prognostic significance in the myelodysplastic syndromes * (Derived from references 34, 38, 42, 43, 68, 84 and 110–121, and other sources.)

	Better prognosis	Intermediate prognosis	Worse prognosis
Clinical features	Younger Female *De novo* MDS		Older (e.g. >60 years) Male Secondary or t-MDS Splenomegaly (in CMML)*
	No transfusion requirement		Requirement for blood transfusion
		Lower number of transfusions per month	Higher number of transfusions per month
FAB category	RA or RARS		RAEB or RAEB-T
WHO category	RA, RARS or 5q– syndrome	RCMD or RAEB-1	RAEB-2 or t-MDS
Peripheral blood features			Anaemia (Hb ≤80, ≤90 or ≤100 g/l) Neutropenia (neutrophil count <0.5, 0.5–1.0, 1–3 or ≤2.5, compared to >2.5 × 10^9/l) Thrombocytopenia (platelet count <20, 20–50, 50–100, 100–150 or >150 × 10^9/l) Presence of blast cells Dyserythropoiesis Dysgranulopoiesis Dysthrombopoiesis Neutrophilia (in CMML)* Monocytosis (in CMML)*† Lymphocyte count less than 1.2 × 10^9/l Presence of CD34-positive cells
Biochemistry	Normal LDH Serum ferritin equal to or less than 1000 ng/ml		Elevated LDH Serum ferritin greater than 1000 ng/ml
Bone marrow aspirate			Increased blast cells (<5%, 5–10%, 10–20%, 20–30%) Increased percentage of CD34-positive cells Dyserythropoiesis Dysgranulopoiesis Dysthrombopoiesis Reduced megakaryocytes
Bone marrow trephine biopsy	Increased mast cells		'Abnormal localization of immature precursors' (ALIP) Increased CD34-positive cells Presence of fibrosis Megakaryocyte atypia Reduced erythropoiesis Increased haemosiderin
Bone marrow culture	Normal numbers of CFU-GM		Reduced numbers of CFU-GM; increased colonies and/or clusters
Cell kinetics			Low labelling index
Ferrokinetics	Near normal iron utilization at 14 days		Low iron utilization at 14 days, increased ineffective erythropoiesis
Karyotype	Normal karyotype	Some normal and some abnormal metaphases	All abnormal metaphases
	del(5q), del(20q), –Y, del(11)(q14q23) and del(12p) as sole abnormalities	trisomy 8 and any other abnormality not associated with good prognosis or poor prognosis	Abnormality of chromosome 7 or both 5 and 7 (but excluding del(7)(q31q35), which is associated with an intermediate prognosis); complex karyotype (e.g. at least three abnormalities in the karyotype), abnormalities of 3q

CFU-GM, colony-forming units – granulocyte, macrophage; FAB, French–American–British (classification); Hb, haemoglobin concentration; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; RCMD, refractory cytopenia with multilineage dysplasia; t-MDS, therapy-related MDS; WHO, World Health Organization. See Table 4.2 for other abbreviation definitions.
* Including FAB-defined as well as WHO-defined categories.
† In some but not all series.

Table 4.7 The International Prognostic Scoring System for myelodysplastic syndrome (MDS) [42].

Prognostic variables	Score 0	0.5	1.0	1.5	2.0
% blasts	<5	5–10	–	11–20	20–30*
Karyotype†	Good	Intermediate	Poor	–	–
Cytopenias‡	0–1	2–3			

Individual scores are summed and cases are then assigned to four risk groups, indicative of an increasingly bad prognosis. A score of 0 is indicative of low risk; a score of 1 is indicative of intermediate risk 1; a score of 1.5–2.0 is indicative of intermediate risk 2; a score of ≥2.5 is indicative of high risk.
* Cases with 20–30% blasts are classified as acute myeloid leukaemia not MDS in the WHO classification.
† Good prognosis karyotype: normal, –Y, del(5q), del(20q). Poor prognosis karyotype: complex (≥3 abnormalities) or chromosome 7 abnormalities. Intermediate prognosis karyotype: other abnormalities.
‡ Cytopenias: Hb <100 g/l, neutrophil count $<1.5 \times 10^9$/l, platelet count $<100 \times 10^9$/l.

Table 4.8 The WHO-classification-based Prognostic Scoring System (WPSS) [51]; individual scores are added together to give four prognostic groups with the clinical relevance as shown in Table 4.9.

Score	0	1	2	3
WHO category	RA, RARS, 5q– syndrome	RCMD, RCMD-RS	RAEB-1	RAEB-2
Karyotype*	Good	Intermediate	Poor	Very poor
Transfusion†	No	Regular	–	–

RCMD, refractory cytopenia with mulilineage dysplasia; RCMD-RS, RCMD and ring sideroblasts; WHO, World Health Organization. See Table 4.2 for other abbreviation definitions.
* Defined as in Table 4.7.
† Defined as having at least one red cell transfusion every 8 weeks over a period of 4 months.

Table 4.9 Outcome in two cohorts of patients according to the WHO-classification-based Prognostic Scoring System (WPSS) [51]; columns 2 and 4 represent the test cohort ($n = 271$) and columns 3 and 5 the validation cohort ($n = 193$).

	Overall survival in months		Percentage of patients showing transformation to AML by 5 years	
Score	$n = 271$	$n = 193$	$n = 271$	$n = 193$
0	103	141	6	3
1	72	66	24	14
2	40	46	48	33
3	21	26	63	54
4	12	9	100	84

AML, acute myeloid leukaemia.

the Lausanne–Bournemouth system [39], the IPSS [42] and the WPSS [51]. Of these, the IPSS [42] (Table 4.7) has been most widely used and validated. The more recently proposed WPSS has likewise been validated in a second cohort of patients [51] and has also been found to retain prognostic significance in patients treated by bone marrow transplantation [123] (Tables 4.8 and 4.9). Modifications of the IPSS cytogenetic classification have also been proposed with del(11)(q14q23) and del(12p)

Table 4.10 The WHO classification of *de novo* myelodysplastic syndromes (MDS) [6,7,124–130].

Disease	Peripheral blood findings	Bone marrow findings
Refractory cytopenia with unilineage dysplasia (RCUD) including refractory anaemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT)	Anaemia or cytopenia (not pancytopenia), blasts rarely seen and always less than 1%	Unilineage dysplasia, <5% blasts, <15% ring sideroblasts
Refractory anaemia with ring sideroblasts (RARS)	Anaemia, no blasts	Dysplasia confined to erythroid lineage, <5% blasts, ≥15% ring sideroblasts
Refractory cytopenia with multilineage dysplasia (RCMD)	Cytopenias (bicytopenia or pancytopenia), no or rare blasts (<1%), no Auer rods, $<1 \times 10^9$/l monocytes	Dysplasia in ≥10% of the cells of two or more myeloid cell lineages, <5% blasts, no Auer rods, ring sideroblasts may be <15% or ≥15%
Refractory anaemia with excess blasts, 1 (RAEB-1)	Cytopenias, <5% blasts, no Auer rods, $<1 \times 10^9$/l monocytes	Unilineage or multilineage dysplasia, 5–9% blasts, no Auer rods
Refractory anaemia with excess blasts, 2 (RAEB-2)*	Cytopenias, 5–19%* blasts, Auer rods sometimes present, $<1 \times 10^9$/l monocytes	Unilineage or multilineage dysplasia, 10–19% blasts*, Auer rods sometimes present
Myelodysplastic syndrome, unclassifiable (MDS-U)	Cytopenias, no Auer rods, ≤1% blast cells (i) Like RCUD or RCMD but 1% blast cells in blood (ii) Like RCUD but pancytopenia (iii) Like RCUD but <10% dysplastic cells in any lineage with myelodysplasia-associated cytogenetic abnormality†	<5% blasts, no Auer rods
MDS associated with isolated del(5q)	Anaemia, platelet count usual normal or elevated, <5% blasts	Megakaryocytes in normal or increased numbers but with hypolobated nuclei, <5% blasts, no Auer rods, del(5q) as sole cytogenetic abnormality

* Assigned to this category if *either* 5–19% peripheral blood blast cells *or* 10–19% bone marrow blast cells *or* Auer rods.
† See Table 4.11.

as single defects being classified as good prognosis, del(7)(q31q35) as intermediate and 3q abnormalities as adverse [84].

The WHO classification of the myelodysplastic syndromes/ neoplasms

The WHO classification is hierarchical, with cases first being assigned to t-MDS and then, if appropriate, to the category of MDS with isolated del(5q). Other cases are then categorized, on the basis of the number of blast cells in the blood and marrow, the presence of unilineage or multilineage dysplasia and the presence or absence of ring sideroblasts. Table 4.10 summarizes the criteria for the WHO categories of *de novo* MDS [6,7,124–130] and Fig. 4.19 shows diagrammatically how cases are assigned to categories. A 500-cell differential count on the bone marrow aspirate and a 200-cell differential count on the blood film are required. Dysplasia is recognized in a lineage if at least 10% of cells are dysplastic. For the megakaryocyte lineage, at least 30 cells must be assessed.

Refractory cytopenia with unilineage dysplasia, including refractory anaemia

The great majority of cases that fall into this group have refractory anaemia (RA) but some have

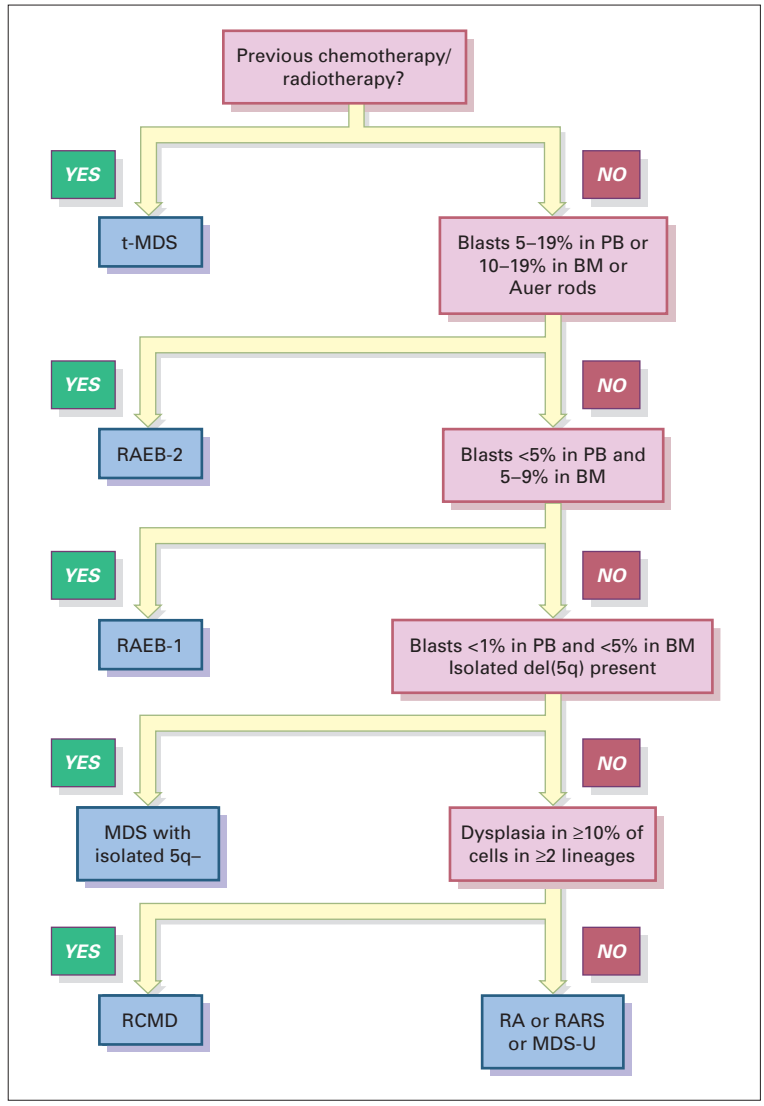


Fig. 4.19 A method of applying the 2008 WHO classification of MDS. For all categories the monocyte count must be less than 1×10^9/l. MDS-U, myelodysplastic syndrome, unclassifiable; t-MDS, therapy-related MDS (for other abbreviations, see earlier captions).

refractory neutropenia (RN) or refractory thrombocytopenia (RT) [124]. The term refractory cytopenia (RC) encompasses all three possibilities. Cytopenia and dysplasia usually affect the same lineage but not necessarily. RA thus defined comprises 5–10% of MDS [43]. Significant dysplasia is confined to one lineage, usually but not necessarily the erythroid lineage. There may be cytopenia affecting two lineages but, by definition, not three. The platelet count is less than 450×10^9/l (or the case would be classified as MDS/MPN not as MDS).

Usually either the patient presents with symptoms of anaemia or the diagnosis is an incidental one. A small minority of patients have hepatomegaly or splenomegaly.

There is anaemia (or other cytopenia) and in anaemic patients an inappropriately low reticulocyte count. In RT, thrombocytopenia may be cyclical [23]. In some cases of RT, platelet lifespan is somewhat reduced but thrombocytopenia is consequent mainly on ineffective production of platelets.

The peripheral blood film may show mild to marked anisocytosis and poikilocytosis. Cells are usually normochromic and either macrocytic or normocytic, but occasional cases have a population of hypochromic cells. In macrocytic cases, the degree of anisocytosis is less than that which is seen in megaloblastic anaemia and oval macrocytes are not usual. There may be basophilic stippling. Cases of RN usually have neutrophil dysplasia and patients with RT may have large or hypogranular platelets. Blast cells are uncommon and are, by definition, always less than 1%.

The bone marrow is usually hypercellular but may be normocellular or hypocellular. Erythropoiesis is dysplastic and either normoblastic, macronormoblastic or megaloblastic. Erythroid dysplasia varies from slight to moderate. Rarely there is a red cell hypoplasia or aplasia [131] (see Fig. 4.2). Such cases have, in the past, sometimes been misdiagnosed as pure red cell aplasia; their recognition as part of the spectrum of MDS is important. Ring sideroblasts may be present but are less than 15% of erythroblasts. Iron stores are often increased. Blast cells are less than 5% of nucleated cells. Cases of RN usually have granulocyte dysplasia. Cases of RT generally have megakaryocyte dysplasia, e.g. micromegakaryocytes.

A clonal cytogenetic abnormality is found in 40–50% of patients with RA, most often del(20q), trisomy 8 or abnormalities of 5, 7 or both [84,124]. However, cases with an isolated del(5q) are excluded from this category. RT is preferentially associated with isolated del(20q), usually del(20)(q11.2) [86]; the dysplasia in these cases may be confined to the erythroid lineage and is not marked [86].

Refractory anaemia shows a low rate of evolution to AML. In one series only 6% of patients developed AML and the median survival was 66 months [43].

Refractory anaemia with ring sideroblasts

Refractory anaemia with ring sideroblasts (RARS) [125] (see Table 4.10) has ring sideroblasts constituting 15% or more of bone marrow erythroid cells. Ring sideroblasts are defined as erythroblasts with at least a third of the nucleus encircled by five or more siderotic granules, demonstrated with an iron stain. Such cases comprise about 11% of cases of MDS [43]. Significant dysplasia is confined to the erythroid lineage, i.e. other lineages show no more than 10% of cells with dysplastic features.

Usually either the patient presents with symptoms of anaemia or the diagnosis is made incidentally.

The patient is anaemic with the red cells commonly being macrocytic but sometimes normocytic or microcytic. The mean cell volume (MCV) is usually normal or high but occasionally reduced. The blood film (Figs 4.20 and 4.21) is often dimorphic with a predominant population of normochromic macrocytes and a minor population of hypochromic microcytes. Basophilic stippling may be present. A careful search usually reveals the presence of Pappenheimer bodies (Fig. 4.20). A small number of circulating nucleated red blood cells (NRBC) can

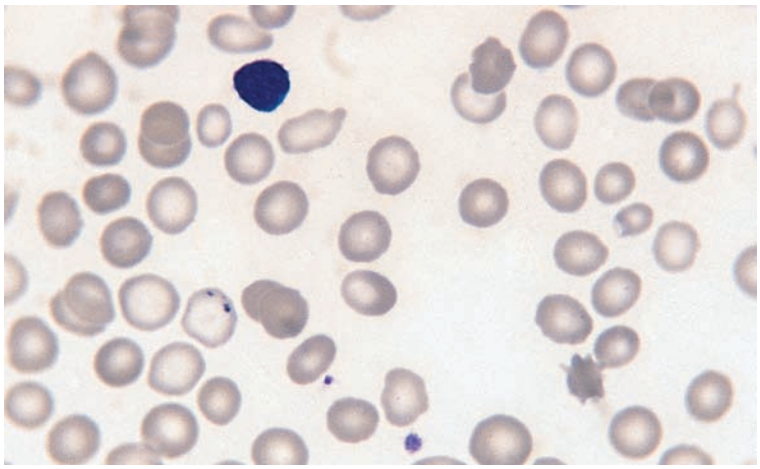


Fig. 4.20 PB film of a patient with RARS. The film is dimorphic and one red cell contains Pappenheimer bodies. Poikilocytes including an acanthocyte are present. Pappenheimer bodies are basophilic iron-containing granules, which can be distinguished from basophilic stippling by being larger, more peripherally situated and less numerous within a cell. MGG ×100.

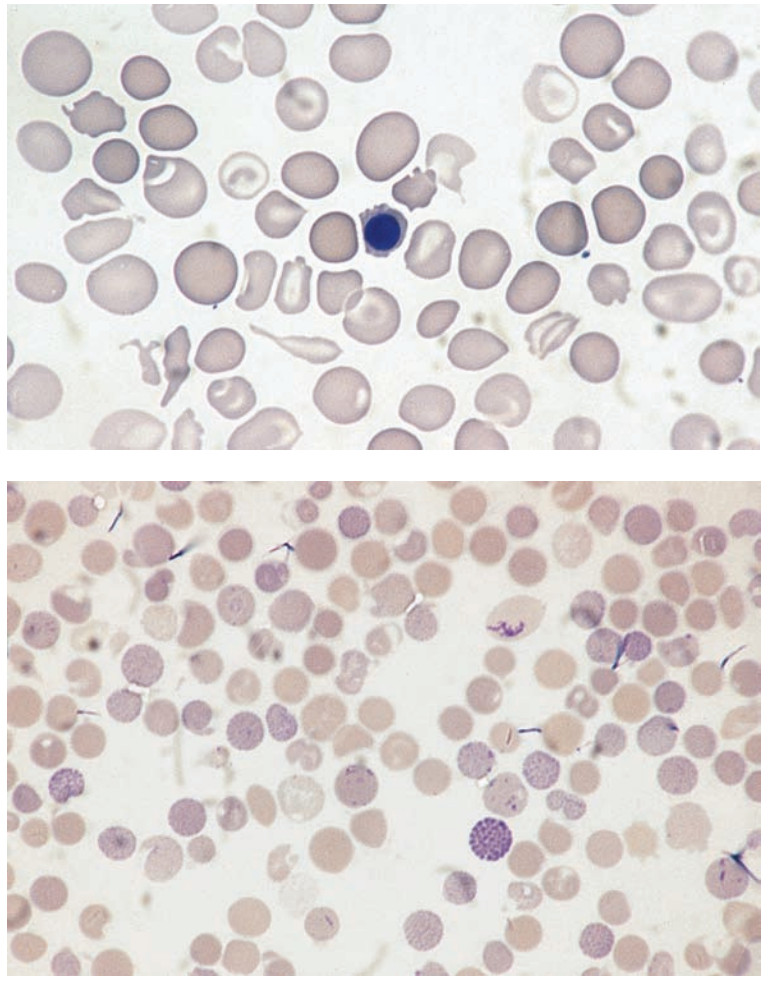
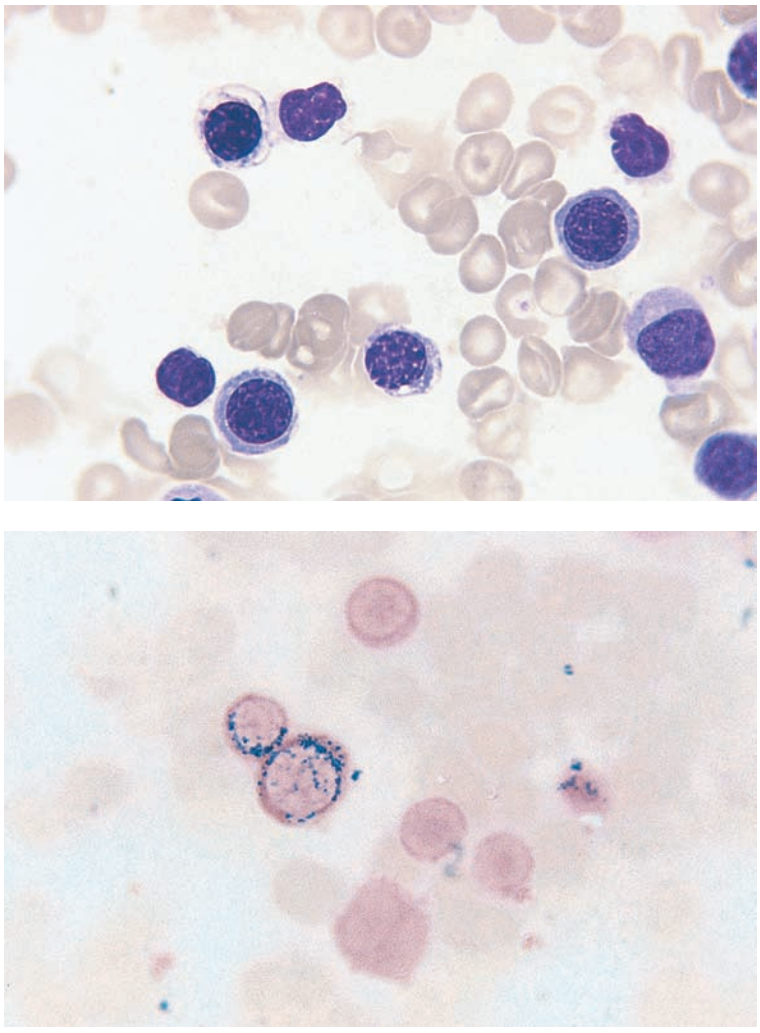


Fig. 4.21 PB film of a patient with RARS with acquired haemoglobin H disease. (a) MGG-stained film showing anisocytosis, poikilocytosis (including target cells), one hypochromic cell and a nucleated red blood cell. (b) Haemoglobin H preparation showing a typical 'golf-ball' cell with haemoglobin H inclusions. MGG ×100. (By courtesy of Dr Jane Mercieca, St Helier.)

often be found and it may be noted that they show defective haemoglobinization and sometimes basophilic cytoplasmic granules adjacent to the nucleus. An iron stain will confirm the nature of Pappenheimer bodies and thus positively identify siderocytes and ring sideroblasts. There may be neutropenia or thrombocytopenia but, by definition, not both. There is no significant neutrophil or megakaryocyte dysplasia. The platelet count may also be increased but is less than 450×10^9/l; a higher count would lead to the case being classified as MDS/MPN, specifically as RARS with thrombocytosis (RARS-T). According to the WHO criteria there are no circulating blast cells. In the experience of the author, occasional blast cells may be present but they are less than 1%. The monocyte count does not exceed 1×10^9/l.

The bone marrow is generally hypercellular with erythroid hyperplasia and dyserythropoiesis. Erythropoiesis may be normoblastic, macronormoblastic or megaloblastic with an appreciable percentage of erythroblasts showing either ragged, scanty cytoplasm or more ample cytoplasm that is defectively haemoglobinized and contains granules (Fig. 4.22a). Ring sideroblasts are readily identified on an iron stain (Fig. 4.22b). There is also an increase of other abnormal sideroblasts in which the iron-containing granules are increased in size and number but are not disposed in a ring. In RARS, siderotic granules are often present in early



(a)

(b)

Fig. 4.22 BM aspirate films of a patient with RARS. (a) Erythroid hyperplasia with only slight dyserythropoiesis. One erythroblast shows defective haemoglobinization and has basophilic granules within its cytoplasm. MGG ×100. (b) Perls' reaction for iron showing two ring sideroblasts. Perls' stain ×100.

erythroid cells from basophilic erythroblasts onwards whereas in secondary sideroblastic anaemia the changes may be confined to late erythroblasts. Ultrastructural examination shows that in ring sideroblasts the iron is deposited in mitochondria whereas in most other abnormal sideroblasts and in the sideroblasts of normal bone marrow the iron is in cytoplasmic micelles. A silver stain has been found to be more sensitive than a Perls' stain in the detection of ring sideroblasts, particularly in patients with low or absent iron stores [132]. Iron-laden macrophages may be prominent. Increased iron stores in advance of any transfusion therapy may be a feature of other categories of MDS but it is most common in sideroblastic anaemia. If a patient with sideroblastic anaemia develops coincidental severe iron deficiency the percentage of ring sideroblasts falls in most but not all cases and rarely ring sideroblasts totally disappear only to reappear when iron stores are replenished.

Clonal cytogenetic abnormalities have been reported in 5–30% of patients [84,128]. Cases with an isolated del(5q) are excluded from the RARS category. One patient has been described with acquired erythropoietic porphyria associated with (probably radiation-induced) RARS [133]. In another

patient, an acquired sideroblastic anaemia was found to be the result of a somatic mutation in the *ALAS2* gene, encoding ALA synthase 2 [134]; since the cells with the somatic mutation replaced normal haemopoietic cells it appears appropriate to regard this patient as having MDS.

RARS shows a very low rate of evolution to AML. In one series 1.4% of patients developed AML and the median survival was 69 months [43].

Refractory cytopenia with multilineage dysplasia

The refractory cytopenia with multilineage dysplasia (RCMD) category, which now encompasses cases with and without a minimum of 15% ring sideroblasts [126], comprises 30–40% of cases of MDS [43]. There is bicytopenia or pancytopenia with dysplastic features in 10% or more of cells in at least two lineages.

There is anaemia and neutropenia, thrombocytopenia or both. In addition to red cell abnormalities (Fig. 4.23), neutrophils and platelets may show dysplastic features such as the pseudo-Pelger–Huët anomaly (see Fig. 4.1) or hypogranular neutrophils or large or agranular platelets. Peripheral blood blasts are less than 1% and monocytes are less than 1×10^9/l.

The bone marrow is usually hypercellular with bilineage or trilineage dysplasia (Figs 4.24–4.26; see also Fig. 4.2). Blasts are less than 5% and ring sideroblasts may be present. Clonal cytogenetic abnormalities are present in half to three quarters of patients and may be complex [84,126].

In one series of patients, median survival in those without 15% ring sideroblasts was 33 months with 10% transformation to AML [43], and in those with at least 15% ring sideroblasts the median survival was 32 months with 13% transformation to AML [43].

Refractory anaemia with excess of blasts

The WHO classification divides refractory anaemia with excess of blasts (RAEB) into two categories, RAEB-1 and RAEB-2, on the basis of blast numbers [127]. RAEB-1 has 2–4% circulating blasts, 5–9% bone marrow blasts or both; Auer rods are absent. RAEB-1 comprises around a fifth of cases of MDS. RAEB-2 is diagnosed when there are *either* 5–19% peripheral blood blast cells *or* 10–19% bone marrow blast cells *or* Auer rods. In one series RAEB-2 comprised 18.5% of cases of MDS [43].

Patients usually present with symptoms of anaemia or with infection, bruising or haemorrhage.

There is anaemia; neutropenia and thrombocytopenia are also common. The monocyte count does not exceed 1×10^9/l. Bilineage or trilineage dysplasia is usual [43] (Figs 4.27 and 4.28). There may be ring sideroblasts or Auer rods (RAEB-2).

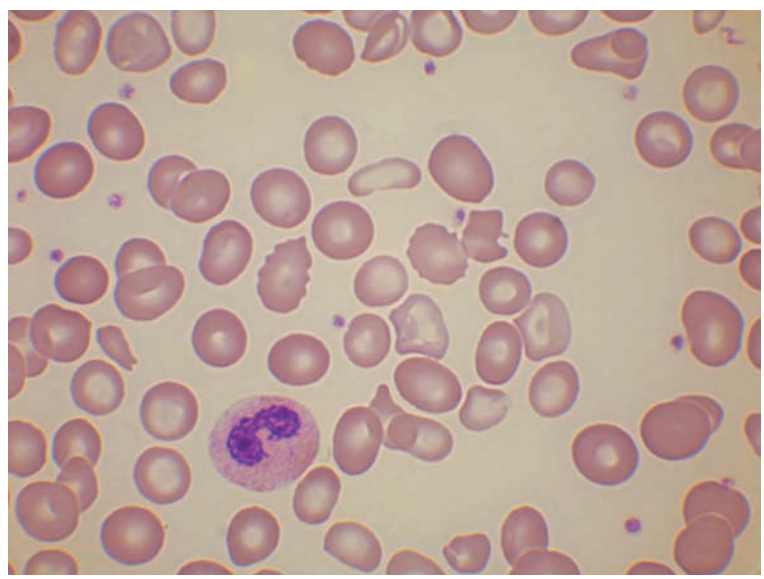


Fig. 4.23 PB film from a patient with refractory cytopenia with multilineage dysplasia (and ring sideroblasts) (RCMD-RS), showing a dysplastic neutrophil, macrocytosis and several hypochromic cells. MGG ×100.

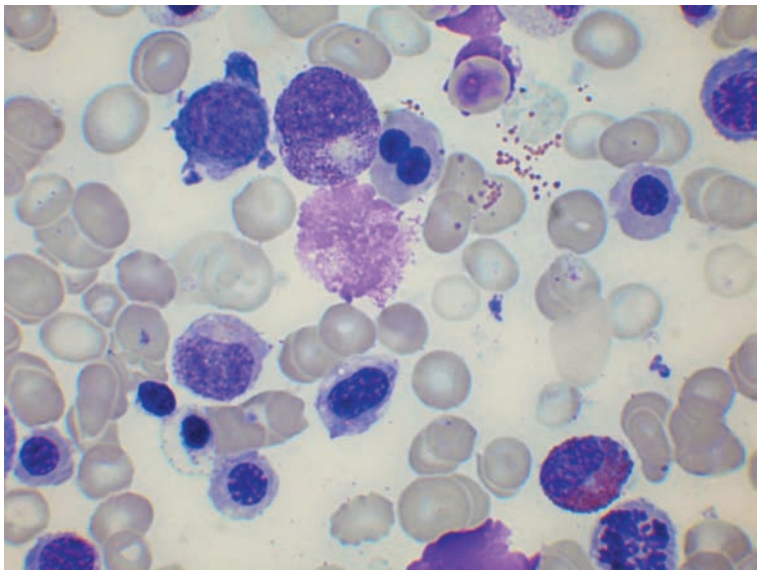


Fig. 4.24 BM aspirate film of a patient with RCMD-RS, showing erythroid dysplasia; there is one erythroblast with very scanty cytoplasm (bottom left) and immediately adjacent to it another with almost empty cytoplasm and basophilic granules, some in juxtaposition to the nuclear membrane (likely to be a ring sideroblast). MGG ×100.

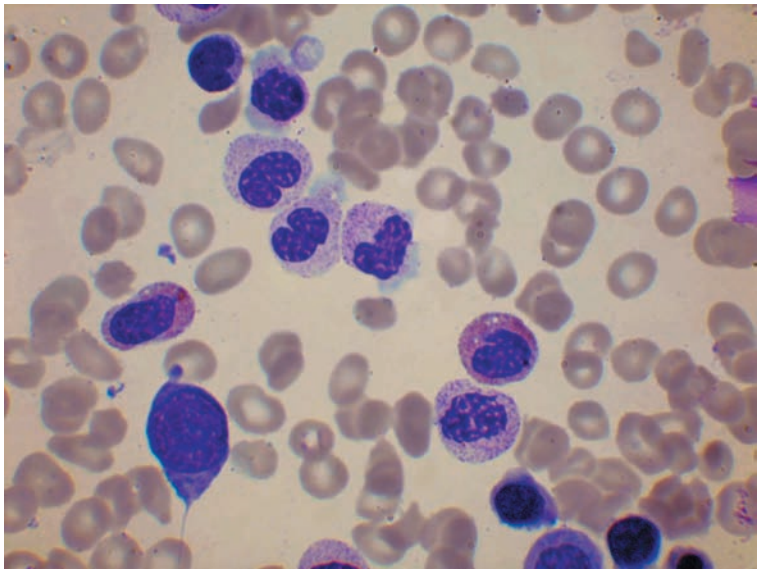


Fig. 4.25 BM aspirate film of a patient with RCMD showing one blast cell and a striking pseudo-Pelger–Huët anomaly affecting both neutrophil and eosinophil lineages. MGG ×100.

In patients with iron overload, haemosiderin granules are sometimes observed in plasma cells (Fig. 4.29). Significant reticulin deposition occurs in about 15% of cases.

Clonal cytogenetic abnormalities, including trisomy 8, abnormalities of chromosomes 5 and 7 and complex karyotypes are present in 50–70% of patients, being more frequent in RAEB-2 than RAEB-1 [84].

Transformation to AML occurs in about a fifth of patients, the others dying of the effects of bone marrow failure. A worse prognosis than non-RAEB categories and a better prognosis of RAEB-1 than RAEB-2 has been confirmed [51,52]. In one large study the median survival was 16 months in RAEB-1 in comparison with 9 months for RAEB-2 ($P = 0.0031$) [135]. Similarly, 22% of patients with RAEB-1 suffered transformation to

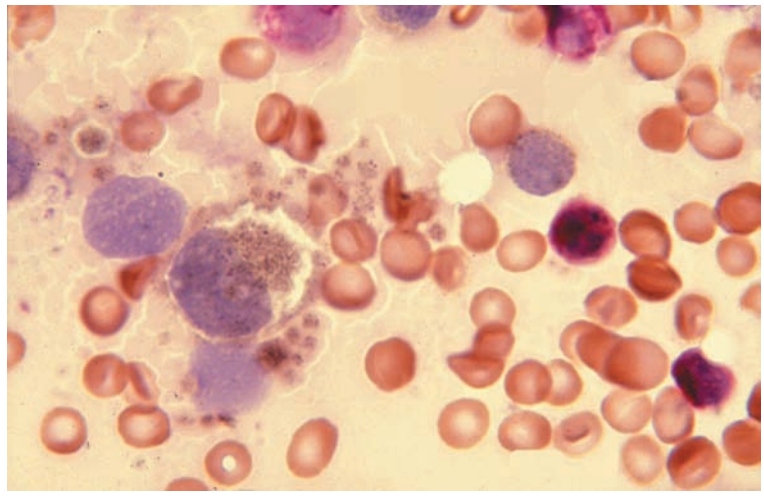


Fig. 4.26 BM aspirate film of a patient with RCMD showing platelets, a micromegakaryocyte (left) and a megakaryoblast (right) that are expressing non-specific esterase (brown reaction product); cells of granulocyte lineage are expressing chloroacetate esterase (red reaction product). Double esterase reaction. MGG ×100.

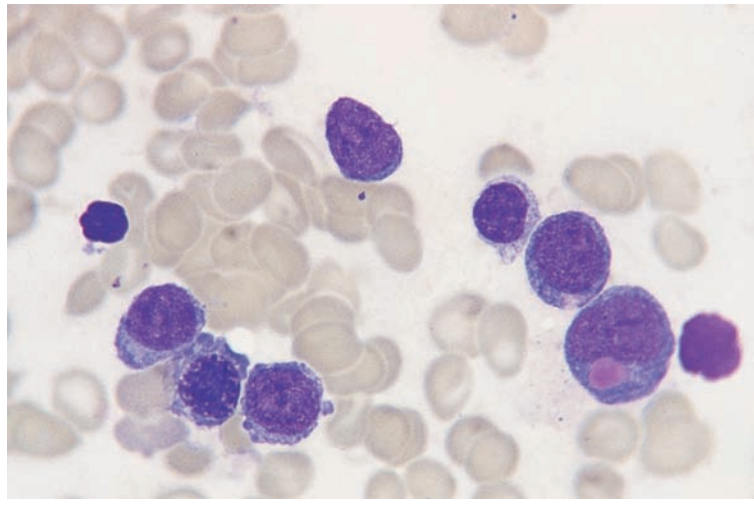


Fig. 4.27 BM aspirate film of a patient with RAEB showing increased, relatively small blasts and a myeloid precursor with a pseudo-Chédiak–Higashi granule. MGG ×100.

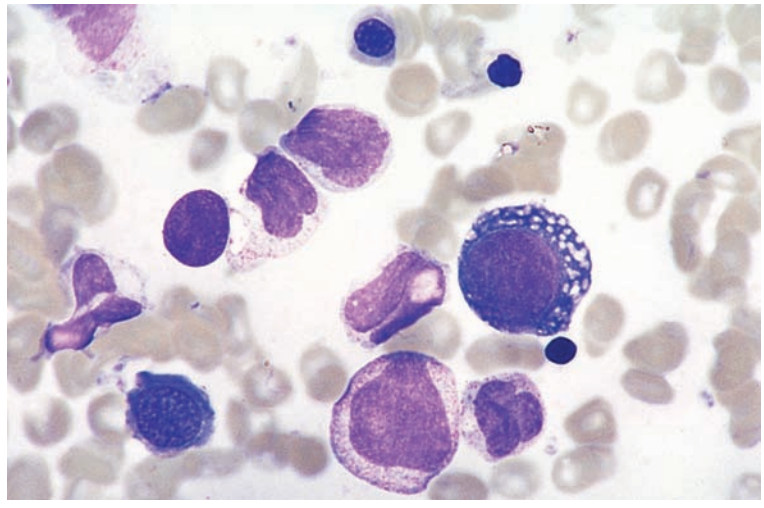


Fig. 4.28 BM aspirate film from a patient with RAEB showing a blast and a heavily vacuolated red cell precursor. MGG ×100.

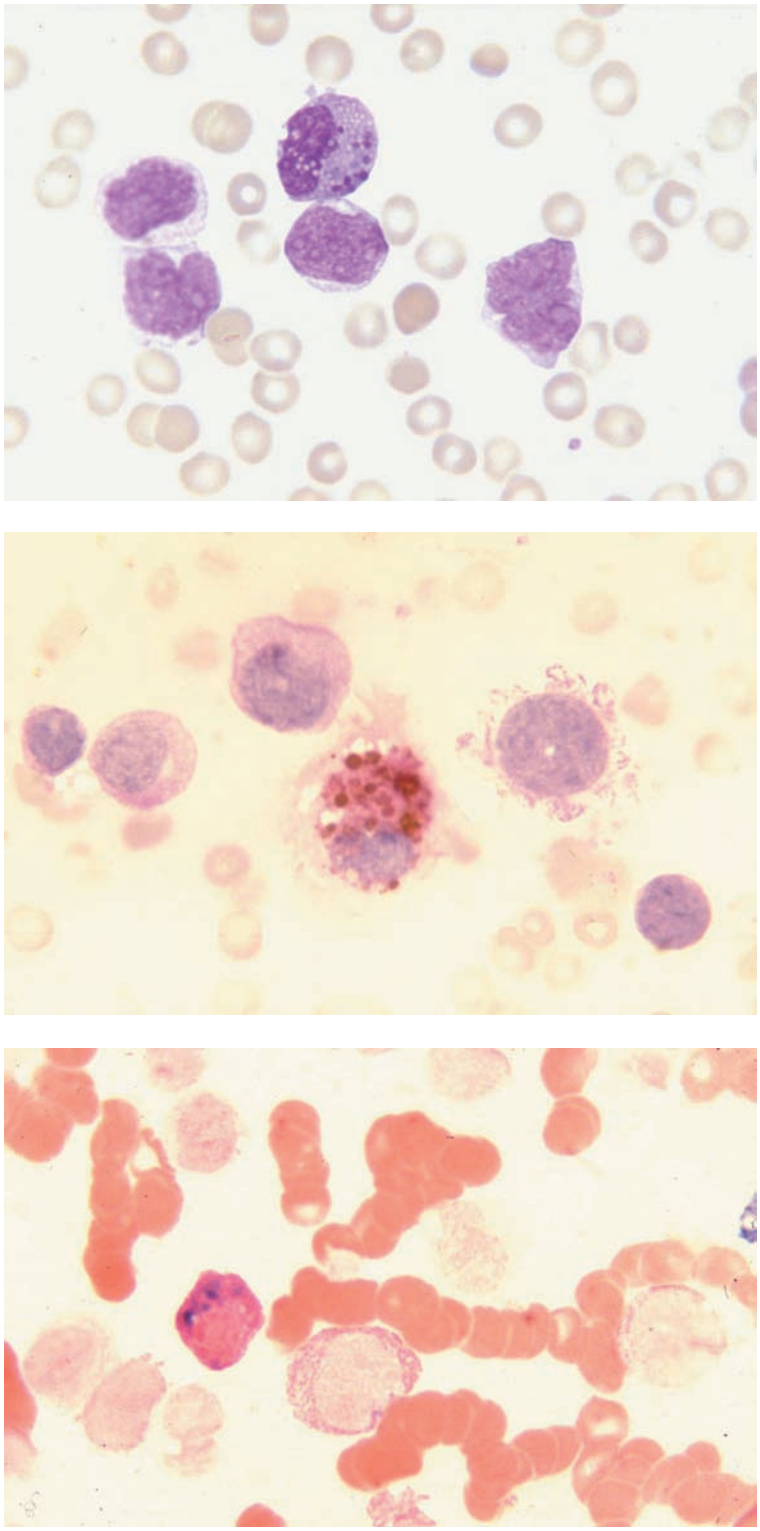


Fig. 4.29 BM aspirate film from a patient with RAEB-2 showing: (a) four blast cells and a plasma cell with vacuoles and haemosiderin deposits; (b) a plasma cell with golden brown haemosiderin deposits – above this plasma cell is a blast cell and to the right of it is a micromegakaryocyte or megakaryoblast with cytoplasmic extensions; (c) haemosiderin deposits in a plasma cell, confirmed by this stain for iron. (a) MGG ×100. (b) Periodic acid–Schiff (PAS) ×100. (c) Perls' stain ×100.

AML in comparison with 40% of patients with RAEB-2 [135]. The likelihood of acute transformation appeared to be higher (48%) in those in whom the diagnosis of RAEB-2 was made on the basis of more than 5% peripheral blood blasts or the presence of Auer rods [135]. Survival in RAEB-2 appears to be better in those who are treated with intensive chemotherapy (28 months compared to 9 months) but it should be noted that no randomized comparison has been made and intensive treatment is only likely to be carried out in younger patients in whom the prognosis with non-intensive treatment is also better [135].

Myelodysplastic syndrome associated with isolated del(5q) ('5q– syndrome')

Deletion of part of the long arm of chromosome 5 (del(5q) or 5q–) was the second recurrent cytogenetic abnormality for which an association with a specific human neoplasm was recognized [136–139]. The clinical and haematological features are sufficiently consistent that the WHO classification recognizes this as an entity [128]. As defined in the WHO classification [128], this is MDS associated with an isolated del(5q) with peripheral blood blast cells being less than 1% and bone marrow blasts being less than 5%.

Patients are mainly women, usually middle aged or elderly. The incidence of MDS with del(5q) (which is not necessarily synonymous with this WHO category) has been estimated at 0.06/100 000/year [9]. The peripheral blood usually shows a macrocytic anaemia (Fig. 4.30), sometimes with thrombocytosis. A minority of patients present with thrombocytosis without anaemia. The white blood cell count (WBC) may be reduced.

The bone marrow may be hypercellular as a result of erythroid hyperplasia but, unusually for MDS, erythropoiesis is often reduced and in a minority of patients erythroid hypoplasia is marked [140]. Erythropoiesis is dysplastic and may be sideroblastic. Megakaryocytes are present in normal numbers or are increased and are cytologically abnormal. They have non-lobed or bilobed nuclei but are mainly more than 30–40 μm in diameter [138] (Fig. 4.31); they thus differ from the mononuclear and binuclear micromegakaryocytes (see Fig. 4.1) associated with other forms of MDS, which are no larger than other haemopoietic cells.

The 5q– syndrome is associated with a variety of interstitial deletions, among which del(5)(q13q33) is prominent. Both the proximal and the distal breakpoints vary with 5q31-32 being the common deleted band. Various candidate tumour suppressor genes that may be lost have been proposed. However, the most promising candidate gene is *RPS14* at 5q32. It appears that haploinsufficiency may be responsible for the phenotype. Other genes in the

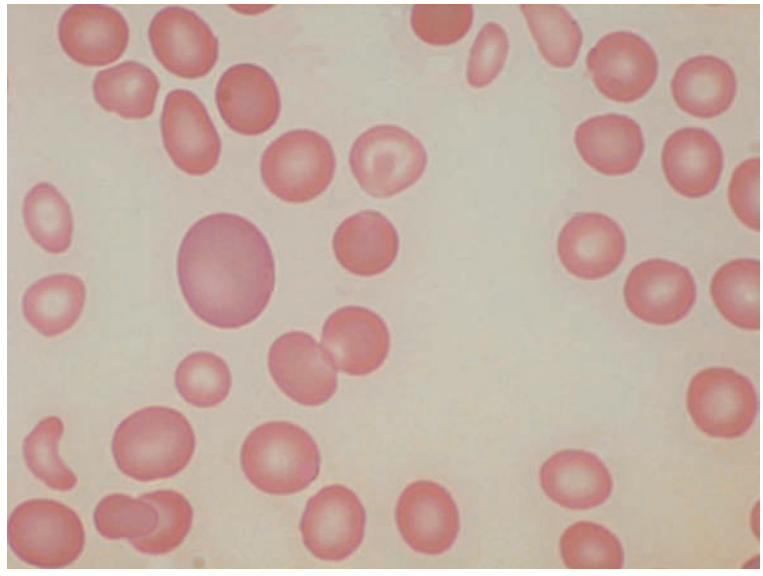


Fig. 4.30 PB film of a patient with the 5q– syndrome showing macrocytes and one target cell. MGG ×100.

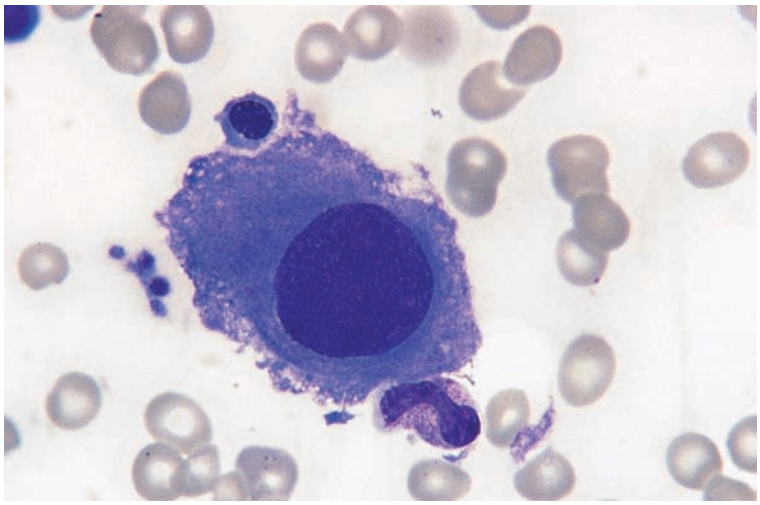


Fig. 4.31 BM aspirate film from a patient with the 5q– syndrome showing a megakaryocyte of normal size but with a hypolobated nucleus. This type of dysplastic megakaryocyte rather than a micromegakaryocyte (see Fig. 4.2) is characteristic of this syndrome. MGG ×100.

common deleted region include *SPARC*, *RBM22*, *CSNK1A1* and *EGR1* [141–143]. CD34-positive cells of the 5q– syndrome have a characteristic gene expression profile that differs from that of refractory anaemia with a normal karyotype; there is a defect in the expression of genes concerned with ribosome formation and control of translation [144]. A minority of patients with the 5q– syndrome have a *JAK2* V617F mutation [145].

In one series of patients classified largely according to the WHO criteria, the 5q– syndrome comprised around 2% of patients [43]. The rate of leukaemic transformation was 3% and the median survival was 116 months. In this series, a small number of patients with more than 5% bone marrow blasts had a strikingly worse prognosis [43]; such patients were excluded from the WHO classification as finally published.

Studies using FISH show that, although morphological abnormalities may be confined to the erythroid or erythroid and megakaryocyte lineages, this is a trilineage disorder [139]. The detection rate of 5q– is significantly increased by the use of FISH.

This form of MDS is particularly susceptible to treatment with the thalidomide analogue, lenalidomide [146]. Many patients become transfusion independent and some actually become polycythaemic and need venesection [143]. A partial or complete cytogenetic response is common and elevated blast cell counts often fall [143]. Responses to this drug also occur in patients with del(5q) who do not meet the criteria for the 5q– syndrome but these responses tend to be less well sustained [143].

Myelodysplastic syndrome, unclassifiable

The WHO classification recognizes that there were some cases of MDS that do not fit the categories described above but who nevertheless do have MDS. This category of MDS, unclassifiable (MDS-U), is heterogeneous [129]. There are three groups of patients assigned to this category because their prognoses may differ from the defined groups to which they are most similar: (i) resembling refractory cytopenia with unilineage dysplasia (RCUD) but with 1% blast cells in the blood; (ii) resembling RCUD but with pancytopenia rather than unilineage or bilineage cytopenia; and (iii) having persistent cytopenia with 1% or fewer blasts in the blood and fewer than 5% in the bone marrow and unequivocal dysplasia in less than 10% of cells in one or more myeloid lineages but with cytogenetic abnormalities considered as presumptive evidence of MDS [7] (Table 4.11). Clonal cytogenetic abnormalities were detected in 57% of patients with MDS-U, as defined in the 2001 WHO classification [84].

In addition, there are small numbers of patients with macrocytosis [147] or sideroblastic erythropoiesis [148] who have clonal haemopoiesis but are not anaemic and do not meet the other criteria for inclusion in the WHO categories of MDS. Assigning such cases of ‘refractory macrocytosis’

Table 4.11 Cytogenetic abnormalities providing presumptive evidence of MDS in patients with persistent cytopenia but insufficient dysplasia to make a morphological diagnosis; abnormalities are shown in the order of their frequency in *de novo* MDS [7].

–7 or del(7q)
–5 or del(5q)
i(17q) or t(17p)
–13 or del(13q)
del(11q)
del(12p) or t(12p)
del(9q)
idic(X)(q13)
t(1;3)(p36.3;q21.2)
t(2;11)(p21;q23)
inv(3)(q21q26.2)
t(6;9)(p23;q34)

and 'refractory sideroblastic erythropoiesis' to the MDS-U category might be considered.

Childhood myelodysplastic syndromes

Children suffer from a range of myelodysplastic and myelodysplastic/myeloproliferative neoplasms that differ from those of adults. If children who have been exposed to cytotoxic chemotherapy and those with genetic disorders such as Down syndrome, Noonan syndrome and neurofibromatosis are excluded, such disorders are much less common in children than in adults, the overall incidence being about 1.2 cases/1 000 000/year [149]. The usual haematological characteristics differ from those in adults. RARS is very rare in childhood and, if this diagnosis appears possible, alternative diagnoses such as congenital sideroblastic anaemia or a mitochondrial cytopathy such as Pearson syndrome must be considered. The 5q– syndrome is also very rare. RA is likewise uncommon so that generally MDS is of higher grade in children than in adults. However, children whose disease meets the FAB criteria for RAEB-T (20–29% bone marrow blast cells) may have a more slowly progressive disease than RAEB-T in adults. These particular features of childhood MDS have led to the inclusion in the 2008 WHO classification of a specific category for childhood MDS [130]. Within this category a single provisional entity is defined, refractory cytopenia of childhood (RCC), which represents about half of childhood cases of MDS. Other children have RAEB-1 or RAEB-2.

Refractory cytopenia of childhood leads to neutropenia in a quarter of the patients, anaemia in about half and thrombocytopenia in about three quarters [130]. There are fewer than 2% circulating blast cells and fewer than 5% bone marrow blast cells. Macrocytosis is common and the percentage of haemoglobin F may be increased. The bone marrow is hypocellular in three quarters of patients so that congenital and acquired aplastic anaemia is included in the differential diagnosis and the trephine biopsy is important for diagnosis.

Monosomy 7 is present in about 10% of patients and is predictive of disease progression [149]. The other most frequently observed cytogenetic abnormalities are trisomy 8 and trisomy 21.

Therapy-related myelodysplastic syndrome

Therapy-related MDS has distinctive haematological and cytogenetic features. Marked trilineage dysplasia is common, even when there is no increase in blast cells (see Fig. 4.1). Eosinophilia and basophilia are more common than in *de novo* cases. Hypocellularity and reticulin fibrosis are common. Cytogenetic abnormalities are more often present than in *de novo* MDS and often include particularly adverse abnormalities such as abnormalities of both chromosomes 5 and 7 and complex karyotypic abnormalities. There are also several translocations, e.g. t(11;16)(q23;p13.3) and t(3;21)(q26.2;q22.1), that are specifically associated with therapy-related disease. t-MDS has a high rate of leukaemic transformation and a shorter survival than *de novo* disease. The prognosis is similar to that of therapy-related AML, with which it is grouped in the WHO classification [150]. The prognosis is uniformly poor, regardless of the blast percentage or degree of dysplasia, although it is worse if there is a complex karyotypic abnormality [151].

Other categories of myelodysplastic syndromes

Hypocellular myelodysplastic syndrome

The FAB group initially described MDS as having a hypercellular or normocellular bone marrow. Subsequently it became apparent that some cases

from all FAB categories, approximately 10% in all, had a hypocellular bone marrow. Their prognosis does not differ from MDS in general [152] and although it is necessary to recognize these cases as MDS it does not appear to be important to assign them to a separate category. The differential diagnosis of hypocellular MDS includes hypocellular AML, aplastic anaemia and autoimmune myelopathy. It is not yet clear whether cases with a severely hypocellular bone marrow and a clonal cytogenetic abnormality but without morphological dysplasia should be classified as MDS or as aplastic anaemia. Although such cases clearly have a neoplastic clone of cells in the bone marrow the clinical course may be that of aplastic anaemia [153].

Myelodysplastic syndrome with myelofibrosis

Some patients with MDS have considerable reticulin deposition. It is important to distinguish these cases from acute megakaryoblastic leukaemia with myelofibrosis and from acute panmyelosis, both of which have more blast cells (and, in the case of acute panmyelosis, also more proerythroblasts) than MDS with fibrosis. Patients with MDS and myelofibrosis have a high incidence of complex chromosomal abnormalities and a poor prognosis [38]. As long as a bone marrow aspirate adequate for diagnostic purposes is obtained they can be assigned to a WHO category. However, the aspirate is often inadequate and examination of trephine biopsy sections with immunohistochemistry for CD34 is an important part of the assessment. In view of the worse prognosis, the presence of reticulin fibrosis should be noted.

Conclusions

The classification of MDS remains largely morphological with refinements being directed at defining categories that can be related to prognosis. The recognition of MDS with isolated del(5q) is a first step towards a molecular classification, this category having both prognostic and therapeutic relevance. Since MDS is highly heterogeneous and molecular changes are very complex it is likely that further advances in classification and any subsequent impact of classification on patient management will be slow.

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FIVE

CHRONIC MYELOID LEUKAEMIAS

Introduction

The World Health Organization (WHO) classification assigns some chronic myeloid leukaemias to the category of myeloproliferative neoplasm (MPN) and others, in which there are also dysplastic features, to the category of myelodysplastic/myeloproliferative neoplasm (MDS/MPN) [1]. In addition, some chronic leukaemias with prominent eosinophilic differentiation and specific genetic lesions are categorized on the basis of these genetic changes [2]. The 2008 WHO classification of these various chronic myeloid leukaemias is summarized in Table 5.1 [1–3]. It should be noted that, in the French–American–British (FAB) classification, chronic myelomonocytic leukaemia (CMML) was considered to be one of the myelodysplastic syndromes (MDS) whereas in the WHO classification it is regarded as one of the overlap MDS/MPN. The term 'chronic myeloid leukaemia' is often used to refer to a specific entity associated with the

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Table 5.1 Classification of the chronic myeloid leukaemias, based on the 2008 WHO classification [1–3].

Myeloproliferative neoplasms
Chronic myelogenous leukaemia, Philadelphia chromosome positive (t(9;22)(q34;q11.2), *BCR-ABL1* positive
Chronic neutrophilic leukaemia
Chronic eosinophilic leukaemia, not otherwise specified
Myelodysplastic/myeloproliferative neoplasms
Chronic myelomonocytic leukaemia
Atypical chronic myeloid leukaemia, *BCR-ABL1* negative
Juvenile myelomonocytic leukaemia
Myeloid (and lymphoid) neoplasms with eosinophilia and abnormalities of PDGFRA, PDGFRB or FGFR1
Myeloid (and lymphoid) neoplasms with *PDGFRA* rearrangement
Myeloid neoplasms with *PDGFRB* rearrangement
Myeloid (and lymphoid) neoplasms with *FGFR1* rearrangement

Philadelphia chromosome (see below). It seems preferable that this condition be designated 'chronic granulocytic leukaemia' or 'chronic myelogenous leukaemia' with the term 'chronic myeloid leukaemia' being used as a general term to include all chronic myeloid leukaemias, a use analogous to the use of the term 'acute myeloid leukaemia'. However, unfortunately, the term 'chronic myeloid leukaemia' is often used in both a general and a specific sense, leading to a lack of clarity.

The classification of the chronic myeloid leukaemias is based on peripheral blood differential counts, cytological features in the blood and bone marrow, and cytogenetic and molecular genetic analysis. Bone marrow findings are less useful in diagnosis than the peripheral blood features. Cytochemistry can be of some value if cytogenetic and molecular genetic analysis is not available but otherwise it is redundant. Immunophenotyping is of little value during the chronic phase of these diseases although it may give evidence of dysplastic maturation; during acute transformation it has a role in identifying the lineage of blasts.

Chronic granulocytic leukaemia

Chronic granulocytic leukaemia (CGL) is a disease entity with specific haematological, cytogenetic and molecular genetic features, associated with a Philadelphia (Ph) chromosome and a *BCR-ABL1* fusion gene. Alternative designations are 'chronic myelogenous leukaemia', 'chronic myelogenous leukaemia, *BCR-ABL1*-positive' (the preferred WHO terminology), 'chronic myelocytic leukaemia' and 'chronic myeloid leukaemia'. The designation now most often used is 'chronic myeloid leukaemia' but, since this term can also be used for Ph-negative leukaemias thus generating confusion, the designation 'chronic granulocytic leukaemia' has been retained in this book. The incidence is of the order of 1.6/100 000/year [4] with a median age of onset in the sixth decade and a slight male preponderance. The disease is bi- or triphasic with a chronic and an acute phase and, sometimes, an intervening accelerated phase. Both accelerated phase and blast transformation result from further mutation in a cell within the neoplastic clone, which thereby gains an advantage over other *BCR-ABL1*-positive cells. Occasionally patients treated only with imatinib develop Ph-negative high grade MDS or acute myeloid leukaemia (AML) [5]; the interpretation of this event is more problematical.

The chronic phase of chronic granulocytic leukaemia

Clinical and haematological features

Chronic granulocytic leukaemia is predominantly a disease of adults. The usual clinical presentation is with splenomegaly, hepatomegaly, symptoms of anaemia, and systemic symptoms such as sweating and weight loss. In a survey of 430 patients, presenting symptoms were fatigue and lethargy (33%), bleeding (21%), weight loss (20%), splenic discomfort (19%) and sweats (15%) [6]. The spleen was palpable in 76% (more than 10 cm below the costal margin in 37%) and 16% had purpura. Hepatomegaly was uncommon (2%). In 19% of patients the diagnosis was incidental, made when a blood count was performed for an unrelated reason. Very rarely CGL has developed in a patient with a pre-existing Ph-negative myeloproliferative disorder [7].

The peripheral blood usually shows anaemia and leucocytosis with a very characteristic differential count [8] (Fig. 5.1). The two predominant cell types are the myelocyte and the mature neutrophil (Fig. 5.2). Earlier granulocyte precursors are also

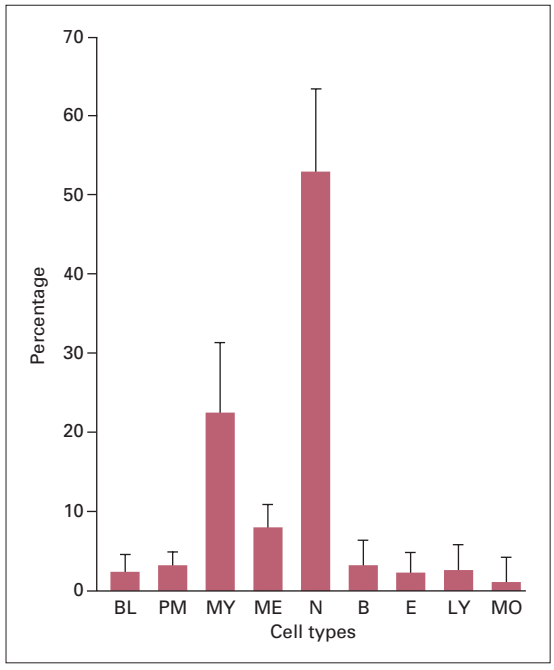


Fig. 5.1 Diagrammatic representation of the typical differential count in 50 untreated cases of chronic granulocytic leukaemia (CGL) (all demonstrated to be Philadelphia (Ph) positive). Each differential count was of 1500 cells. BL, blasts; PM, promyelocytes; MY, myelocytes; ME, metamyelocytes; N, neutrophils; B, basophils; E, nucleated erythroid cells; LY, lymphocytes; MO, monocytes. The mean and the standard deviation are indicated. (Modified from reference 8.)

present but promyelocytes are fewer than myelocytes and blasts are fewer than promyelocytes. Almost all patients have an absolute basophilia and more than 90% have eosinophilia. The absolute monocyte count is increased but not in proportion to the increase in mature neutrophils and the percentage of monocytes is almost always less than 3%. The absolute lymphocyte count can be increased as the result of an increase in T lymphocytes. Occasional nucleated red blood cells and megakaryocyte nuclei may be present. The white blood cell count (WBC) correlates with the degree of splenomegaly and both correlate inversely with the haemoglobin concentration (Hb) [6]. The platelet count is most often normal or somewhat elevated but is low in about 5% of cases. About 1% of patients present with thrombocytosis without leucocytosis [6]. Very rare patients have eosinophilia without neutrophilia [9]. Rarely the Hb is elevated. Dysplastic features are lacking during the chronic phase of the disease. The neutrophil alkaline phosphatase (NAP) score is low in about 95% of patients.

Haematological features differ somewhat between different molecular variants of CGL (see below). The features described above are those seen in the great majority of patients who have a p210 BCR-ABL1 protein. The rare cases with a p190 BCR-ABL1 protein (more characteristic of Ph-positive acute lymphoblastic leukaemia (ALL) than of CGL) have a more prominent relative and absolute monocytosis while those with the very rare p230

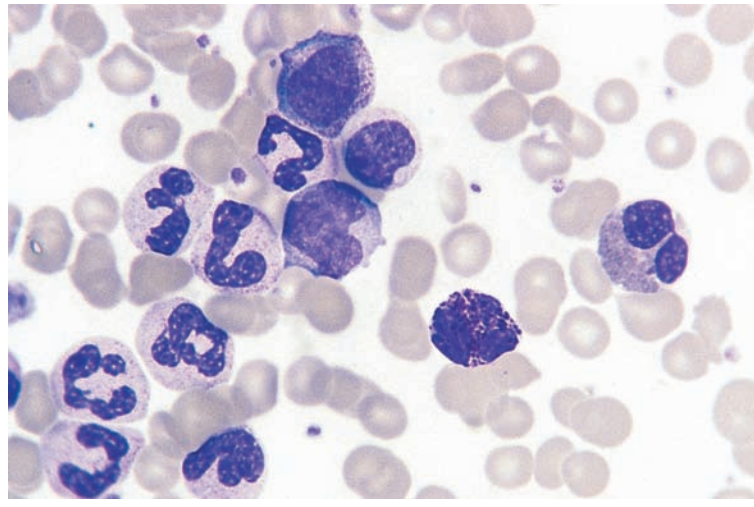


Fig. 5.2 Peripheral blood (PB) film of a patient with CGL showing two promyelocytes, a myelocyte, an eosinophil, a basophil and numerous neutrophils and band forms. May–Grünwald–Giemsa (MGG) ×100.

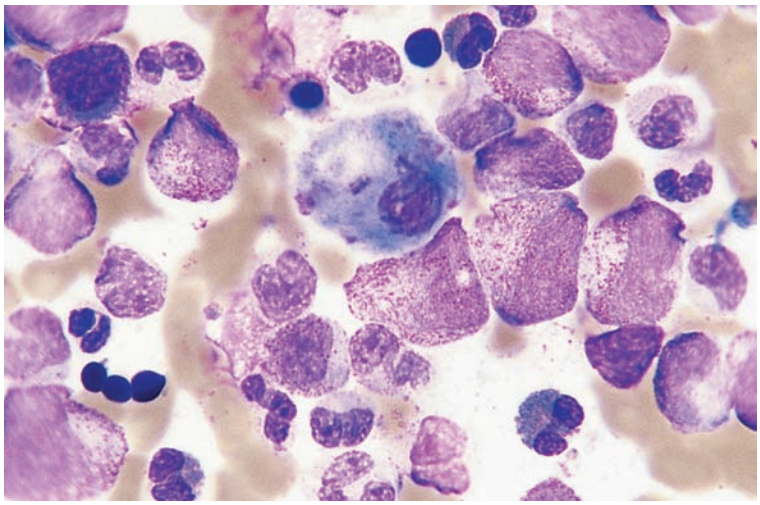


Fig. 5.3 Bone marrow (BM) film of a patient with CGL showing increased granulocytes and precursors and a phagocyte containing cellular debris. MGG ×100.

BCR-ABL1 protein may have a neutrophilic variant of CGL [10].

In untreated CGL, there is usually a progressive rise in the WBC. Rare patients show cyclical changes in the count, e.g. from 30 to 500×10^9/l with a periodicity of about 50 days, suggesting a partially intact negative feedback mechanism [11]; in this patient there was a similar cycle in the platelet count but 1 week in advance of the changes in the WBC [11]. In some patients with cyclical changes, haematological remissions are sometimes prolonged although haemopoietic cells remain Ph positive [12]. The NAP score may cycle inversely to the white cell count [12].

The bone marrow is intensely hypercellular with a marked increase in granulopoiesis (Fig. 5.3) and with the myeloid : erythroid (M : E) ratio being greater than 10 : 1. There is an increase of cells of neutrophil, eosinophil and basophil lineages. Megakaryocytes are either normal in number or increased. Their average size and nuclear lobe count is reduced in comparison with normal megakaryocytes. Pseudo-Gaucher cells and sea blue histiocytes may be increased. Trephine biopsy sections show an increase in cells of all granulocyte lineages and, in some patients, increased megakaryocytes. Rarely the bone marrow shows collagen fibrosis at presentation. An increase of reticulin is much more frequent.

Several staging systems have been proposed for the further categorization of patients with CGL, in order to give an indication of prognosis. The most widely used system is that of Sokal *et al.* [13]. The system of Hasford *et al.* [14] may be more appropriate for interferon-treated patients [15].

Cytogenetic and molecular genetic features

Chronic granulocytic leukaemia was the first malignant disease for which a consistent association with an acquired, non-random, cytogenetic abnormality was recognized. In 1960 Nowell and Hungerford [16] reported its association with an abnormal 'minute chromosome', soon afterwards designated the Philadelphia^1 (Ph^1) chromosome after the city of its discovery. Subsequently it was demonstrated that there was a characteristic translocation, t(9;22)(q34;q11.2), with the derivative chromosome 22q– being the previously reported Philadelphia chromosome [17]. It should be noted that the favoured designation of the abnormal chromosome 22 is now Philadelphia rather than Philadelphia^1 with the abbreviation Ph rather than Ph^1. The 9;22 translocation results in fusion of some of the sequences of the *BCR* (breakpoint cluster region) gene at 22q11 with some of the sequences of the *ABL1* oncogene, which have been translocated from 9q34. A hybrid gene, *BCR-ABL1*, is formed on chromosome 22. *BCR-ABL1* encodes a constitutively activated tyrosine kinase, which is important in leukaemogenesis and is a target for treatment. There is also an *ABL1-BCR* fusion gene on chromosome 9 that is not always transcribed

and, since a protein product has not been identified [18], is unlikely to be relevant to leukaemogenesis. The translocation occurs in a pluripotent stem cell so that the clone of cells with this abnormality includes the granulocytic, monocytic, erythroid and megakaryocytic lineages, and also some precursors of at least B lymphocytes and possibly T lymphocytes.

The typical t(9;22) giving rise to the Ph chromosome is found in about 95% of cases of CGL. A minority of cases have a simple variant translocation (involving either chromosome 9 or chromosome 22 but not both) or a complex variant translocation (with involvement of chromosomes 9, 22 and a third chromosome). Simple variant translocations are actually complex variants that are undetectable as such by standard cytogenetic analysis. Variant translocations may arise by three or more simultaneous chromosome breaks or by two consecutive translocations in quick succession [19]. There are also patients with otherwise typical CGL who demonstrate neither a t(9;22) nor a variant translocation but nevertheless have a molecular rearrangement leading to formation of a *BCR-ABL1* gene. The fusion gene may be at 9q34, at 22q11.2 or on a third chromosome [18,20]. Cases without a Ph chromosome but with *BCR-ABL1* rearrangement are classified as CGL. Their clinical and haematological features, response to treatment and prognosis are identical to those of Ph-positive cases.

At a molecular level, breakpoints in the *BCR* gene vary so that at least three different *BCR-ABL1* fusion genes can occur, leading to the formation of one of three abnormal proteins of different molecular weights designated p210, p190 and p230 [10]. These show different disease associations (Table 5.2). Patients presenting with isolated thrombocythaemia usually have p210.

Fluorescence *in situ* hybridization (FISH) demonstrates that a significant minority of patients with CGL have a large deletion of chromosome 9 material adjacent to the breakpoint on the derivative chromosome 9 [21]. Most patients also have a smaller deletion of chromosome 22 material. This loss of chromosomal material is associated with a neutrophil dysplasia (chromatin clumping, Pelger–Huët anomaly and hypogranularity) [22], resistance to interferon therapy [23] and, in the past, a considerably worse prognosis [21–24]. The adverse effect of deletion of der(9) is confined to those patients in whom the deletion spans the breakpoint [25] and is negated by the use of imatinib therapy [26]. Chromosome 9 deletions appear to occur at the time of the initial translocation and are more common in patients with variant translocations [24]. Most patients with variant translocations have *BCR-ABL1* but not *ABL1-BCR*; in about half of the patients lacking *ABL1-BCR* this is the result of deletions at the breakpoint on the der(9) chromosome [27]. Prior to the availability of imatinib treatment, patients with variant translocations had a worse prognosis than patients with t(9;22), this being entirely the result of the higher frequency of der(9) deletions [27].

There is some evidence that the translocation that leads to *BCR-ABL1* formation may not be the first molecular event in CGL. For example, some patients have a chromosomal abnormality in Ph-negative and Ph-positive cells and sometimes treatment with imatinib leads to disappearance of the cells that have both a Ph chromosome and the unrelated abnormality, leaving only cells with just the unrelated abnormality [28].

Table 5.2 Molecular variants of *BCR-ABL1* and associated clinicopathological features.

Protein	Breakpoint	Disease association
$p210^{BCR\text{-}ABL1}$	M-bcr	The great majority of cases of typical CGL About a third of cases of Ph-positive ALL
$p190^{BCR\text{-}ABL1}$	m-bcr	A minority of cases of CGL, often with monocytosis or dysplastic features About two thirds of cases of Ph-positive ALL Rare cases of AML
$p230^{BCR\text{-}ABL1}$	μ-bcr	Neutrophilic variant of CGL, or CGL with marked thrombocytosis

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia: CGL, chronic granulocytic leukaemia, Ph, Philadelphia chromosome.

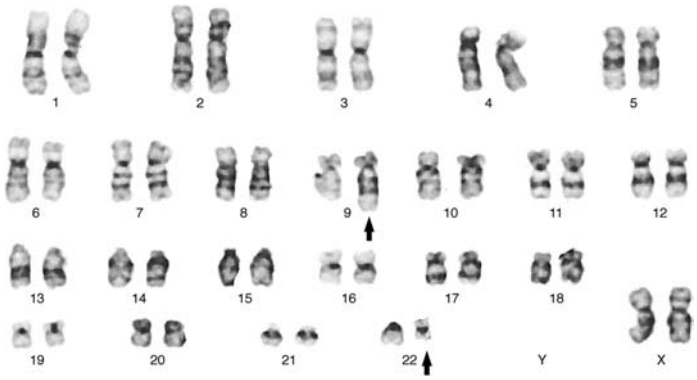


Fig. 5.4 Karyogram of a patient with CGL showing t(9;22)(q34;q11.2). The 22q– derivative chromosome is the Ph chromosome. (By courtesy of Professor Lorna Secker-Walker.)

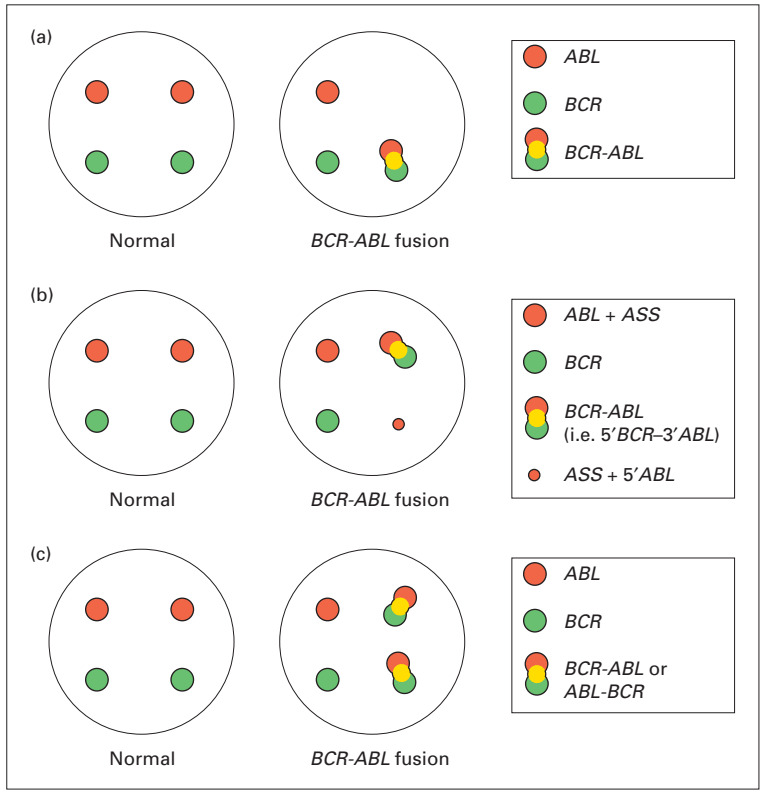


Fig. 5.5 Diagrammatic representation of three fluorescence *in situ* hybridization (FISH) strategies for detection of *BCR-ABL1* fusion. (a) Dual-colour, single-fusion FISH. Normal cells have two red *ABL1* signals and two green *BCR* signals. When t(9;22) is present, there is a red *ABL1* signal, a green *BCR* signal and a yellow *BCR-ABL1* fusion signal. (b) Extra-signal, dual-colour FISH. The *ABL1* probe encompasses also the upstream *ASS* (argininosuccinate synthetase) gene. A normal cell has two red *ASS-ABL1* signals and two green *BCR* signals. When t(9;22) has occurred, there are single normal red and green signals, a yellow fusion signal representing *BCR-ABL1* (i.e. 5′ *BCR*-3′ *ABL*) and a small extra red signal representing *ASS* and 5′ *ABL1*. (c) Dual-colour, dual-fusion FISH. Both probes are split by the t(9;22) translocation when there is a M-bcr breakpoint; normal cells have two red *ABL1* signals and two green *BCR* signals whereas the translocation leads to a cell having single red *ABL1* and green *BCR* signals and two yellow fusion signals representing *BCR-ABL1* and *ABL1-BCR*. The strategies outlined in (b) and (c) increase the specificity of the technique but it should be noted that if there has been a large deletion on the derivative 9, as occurs in a significant minority of patients, the *ABL1-BCR* fusion signal will not be present.

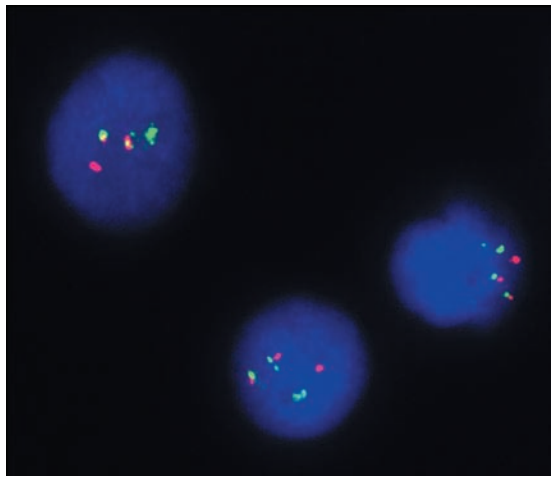


Fig. 5.6 Dual-colour, dual-fusion FISH in CGL. The green probe is for *BCR* on chromosome 22, whilst the red probe (actually Spectrum Orange) is for *ABL1* on chromosome 9. Both probes span their respective breakpoints. In the nucleus of a normal cell, there would be two red and two green signals. In the nucleus of a leukaemic cell such as this one, there is a normal green *BCR* signal, a normal red *ABL1* signal and two fusion red–green signals (fusion of red and green may appear yellow). (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

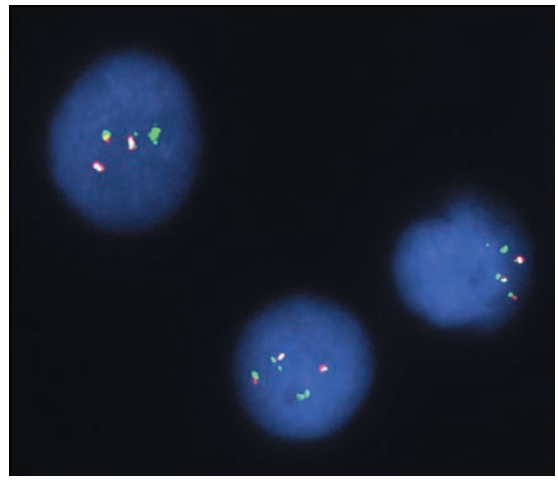


Fig. 5.7 Dual-fusion, tricolour FISH probe in CGL with a supplementary aqua probe (appears pale blue or white) for *ASS*, which is proximal to *ABL1*. The same cells as in Fig. 5.6 have been studied and the *ASS* probe results have been overlayed on the image. The results are those expected with a standard t(9;22) rearrangement. There is a green *BCR* signal on the normal chromosome 22, an aqua–red signal on the normal chromosome 9, an aqua–red–green fusion signal of the derivative chromosome 9 and a red–yellow–green fusion signal on the derivative chromosome 22. No signals have been lost. (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology.)

The characteristic chromosomal rearrangement of CGL can be detected by conventional cytogenetic analysis (Fig. 5.4), FISH, Southern blot analysis for rearrangement of the *BCR* gene (no longer in diagnostic use) and the reverse transcriptase polymerase chain reaction (RT-PCR) for detection of *BCR-ABL1* messenger ribonucleic acid (mRNA). FISH can be performed with a single probe for the *ABL1* gene and a mixture of probes for the *BCR* gene, permitting detection of rearrangements within the major and minor breakpoint cluster regions. The use of two probes for 5′ and 3′ *ABL1* and two probes for 5′ and 3′ *BCR* increases the specificity of FISH (dual-colour FISH). Alternatively, a third probe for the *ASS* gene, adjacent to the *ABL1* gene, can be used to give a triple signal. Various FISH strategies are illustrated in Fig. 5.5. FISH permits the detection of deletions of chromosome 9 or chromosome 22 material, which are not detected by conventional cytogenetic analysis. If dual-colour, dual-fusion FISH is used, the typical pattern is of two single signals representing the two normal genes and two fusion signals representing *BCR-ABL1* and *ABL-BCR1* (Fig. 5.6). Atypical patterns seen include: (i) three fusion signals, representing duplication of the Ph chromosome; (ii) two normal signals and a single fusion signal (likely to represent deletion of *ABL1-BCR* on chromosome 9); (iii) two normal signals, a single fusion signal and loss of either the second *BCR* or the second *ABL1* signal (likely to indicate smaller deletions of material on chromosome 9); and (iv) two *ABL1* signals, two *BCR* signals and a single fusion signal (can occur in a three-way translocation when, instead of *ABL1-BCR* being found on chromosome 9, the parts of the genes usually involved in this fusion gene are on two separate chromosomes). Extra-signal (triple-colour) FISH with a supplementary *ASS* probe is illustrated in Figs 5.7 and 5.8. Patients in whom CGL is suspected should have conventional cytogenetic analysis performed and if a classical t(9;22) is not detected should be further studied by RT-PCR or

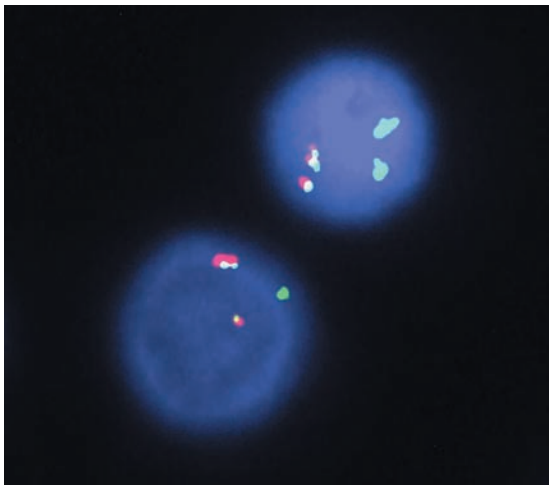


Fig. 5.8 Dual-fusion, tricolour FISH probe in CGL with a supplementary aqua probe (appears pale blue or white) for *ASS*, which is proximal to *ABL1*. The technique is the same as in Fig. 5.7 but illustrates a deletional variant. There is a normal cell (top right) with two green *BCR* signals and two aqua–red *ASS ABL1* signals. The leukaemic cell (bottom left) has a normal green *BCR* signal and a normal aqua–red *ASS ABL1* signal. The derivative chromosome 22 towards the centre of the cell has a red–yellow–green signal. There is no second fusion signal (which would have been aqua–red–green) because the region that includes the proximal *ASS ABL1* and the translocated distal *BCR* has been lost. (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology.)

FISH. Such analysis is also indicated in patients with apparent essential thrombocythaemia with a high basophil count or with unusually small megakaryocytes since such patients may have a *forme fruste* of CGL. Because of the effectiveness of imatinib therapy in patients with *BCR-ABL1* fusion it is justifiable to perform this analysis in *all* patients being investigated for suspected essential thrombocythaemia.

During therapy, CGL can be monitored by conventional cytogenetic analysis, FISH or RT-PCR and its modifications [29]. Cytogenetic analysis has the advantage that clonal evolution will be detected. However, if there is a major cytogenetic response to therapy, the technique is insensitive since conventionally only 20 metaphases are examined. FISH is a more sensitive technique as it is possible to scan many more metaphases. Peripheral blood FISH is a suitable surrogate for bone marrow cytogenetic analysis during follow-up [30]. RT-PCR is more sensitive than FISH and can be made quantitative by use of techniques such as real-time quantitative PCR (RQ-PCR). Neither FISH, RT-PCR nor RQ-PCR permits the detection of secondary cytogenetic abnormalities in the Ph-positive clone nor clonal abnormalities arising in Ph-negative cells. It is important that the specific technique that is to be used for follow-up of minimal residual disease is applied also to the diagnostic sample since deletions of chromosome 9 or 22 material will complicate interpretation of FISH, and RQ-PCR is transcript specific.

Cytogenetic monitoring during follow-up may lead to the detection of clonal evolution within the Ph-positive clone. In addition, when therapy leads to reduction in the size of the Ph-positive clone and reappearance of Ph-negative metaphases, new clonal abnormalities may emerge from among the Ph-negative cells. This has been reported during interferon therapy but appears to be particularly common during imatinib therapy with four instances being observed among 73 patients in two reported series [31,32], 6% of 258 in a third [33] and such changes in as many as 24% of patients achieving a complete or partial cytogenetic remission in a fourth series [34]. The abnormalities observed included those that occur in secondary leukaemias such as del(5q), −7, del(7q), del(13q), +8 and −Y [31,32,34,35]. These clonal abnormalities are not generally accompanied by myelodysplastic features and occasionally they disappear [34]. However they may be the harbinger of development of Ph-negative AML or high grade MDS [5,33,36]. Acute leukaemia associated with inv(16) originating in Ph-negative cells has also been reported [37]. Patients treated with dasatinib can similarly develop clonal cytogenetic abnormalities in Ph-negative cells [38].

Chronic granulocytic leukaemia in accelerated phase and blast transformation

After a variable period in chronic phase, usually several years, CGL undergoes further evolution. There may be an abrupt transformation to an acute leukaemia, designated blast transformation, or there may be an intervening phase of accelerated disease. The International Bone Marrow Transplant Registry has defined criteria for ‘advanced disease’, a similar concept to accelerated phase [39]. In the

WHO classification the following criteria for accelerated phase have been suggested: (i) myeloblasts constituting 10–19% of peripheral blood white cells or bone marrow nucleated cells; (ii) peripheral blood basophils 20% or more of nucleated cells; (iii) persistent thrombocytopenia (platelet count $<100 \times 10^9$/l) that is not a result of treatment or persistent thrombocytosis (platelet count $>1000 \times 10^9$/l) that does not respond to treatment; (iv) increasing WBC and increasing spleen size that does not respond to treatment; (v) cytogenetic evolution; or (vi) marked granulocyte dysplasia or prominent proliferation of small dysplastic megakaryocytes in large clusters or sheets [40]. Whether cytogenetic evolution alone should be regarded as indicative of accelerated disease has been disputed, since patients classified as being in accelerated phase only for this reason appear to have a better prognosis than patients with other features of disease acceleration [3].

Blast transformation may be myeloid or lymphoid. It is important to make the distinction since there is far more chance of a useful response to therapy in a lymphoblastic transformation. Lymphoid blast crisis is more likely to emerge suddenly without a preceding accelerated phase [41]. Transformation may occur initially in the bone marrow or in extramedullary tissues. In the WHO classification the criteria suggested for recognition of blast transformation are: (i) myeloblasts constituting at least 20% of peripheral blood white cells or bone marrow nucleated cells; (ii) extramedullary proliferation of blast cells; or (iii) large aggregates and clusters of blasts in bone marrow biopsy sections [40].

Clinical and haematological features

During the accelerated phase of CGL there may be refractory splenomegaly with recurrence of symptoms present at presentation. The peripheral blood often shows marked basophilia, refractory leucocytosis, anaemia and either thrombocytopenia or marked thrombocytosis. Dysplastic features may appear including hypogranular neutrophils and the acquired Pelger–Huët anomaly of neutrophils or eosinophils.

Blast transformation may be similarly associated with recurrence of fever, weight loss and sweating. In addition there may be bone pain together with lymphadenopathy or other evidence of extramedullary disease. Bruising and bleeding may occur. Increasing numbers of blast cells, out of proportion to the numbers of maturing granulocytic cells, appear in the blood and there may also be circulating micromegakaryocytes and giant dysplastic platelets (Fig. 5.9). A minority of patients have hypogranular neutrophils or the acquired Pelger–Huët anomaly [42]. Blast transformation may be myeloid, lymphoid or mixed phenotype. Myeloid transformation may be multilineage or may be predominately myeloblastic, monoblastic, myelomonocytic, hypergranular promyelocytic, eosinophilic, mast cell and/or basophilic, megakaryoblastic or erythroid. In pure lymphoid blast crisis there are

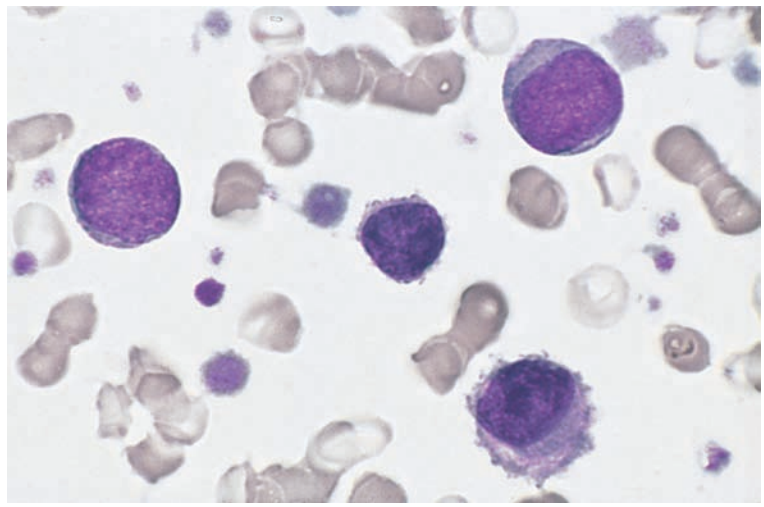


Fig. 5.9 PB film of a patient with megakaryoblastic transformation of CGL showing large platelets, several blast cells and a micromegakaryocyte. MGG ×100.

increasing numbers of blast cells but without the dysplastic features associated with myeloid transformation and without a striking increase in the basophil count. In lymphoid transformation the blasts usually resemble those of the FAB categories of L1 or L2 ALL but rarely they resemble the blasts of L3 ALL [43].

Since the blast cells of acute transformation often show no cytological evidence of differentiation, immunophenotyping may be necessary to confirm their lineage. Lymphoid blast crisis is usually B lineage but occasionally T lineage. B-lineage lymphoid blast crisis may be early precursor B cell or have a common ALL or a pre-B phenotype. Mixed phenotype leukaemias are relatively much more common in CGL in transformation than in *de novo* acute leukaemias.

In the accelerated phase, the bone marrow may show dysplastic features, some increase of blast cells and a striking increase of basophils. With the onset of acute transformation, there are increasing numbers of blast cells, which rapidly replace maturing granulocytic cells. Myeloblasts are usually relatively undifferentiated with few granules. Auer rods are uncommon. Dysplastic features are usually present in various lineages; these may include numerous micromegakaryocytes, and ring sideroblasts are sometimes present. The blast percentage is generally higher in lymphoid transformation [41].

In the accelerated phase, trephine biopsy sections show disorganization and dysplastic features. There may be increasing numbers of blast cells, initially in a paratrabecular and periarteriolar distribution but subsequently also in the intertrabecular space. Following the onset of blast transformation, there is progressive replacement of maturing granulocytic cells by blast cells. In megakaryoblastic transformation there are often also large numbers of dysplastic megakaryocytes, including micromegakaryocytes, occurring in clumps. Reticulin fibrosis is increased. Collagen fibrosis can occur, sometimes with associated osteosclerosis; this is most often associated with myeloid transformation, particularly megakaryocytic/megakaryoblastic transformation. Transformation may be associated with the acquisition of chromosomal rearrangements typical of acute leukaemia and in these cases the cytological findings characteristic of the rearrangement are often present.

Cytogenetic and molecular genetic features

Additional cytogenetic abnormalities often develop several months before the development of blast crisis. The commonest abnormalities, in order of frequency, are +8 (34%), +Ph (31%), i(17q) (21%), +19 (13%), −Y (9% of males), +21 (7%), +17 (6%) and −7 (5%), followed by −17, +6, +10 and +14 (all between 3% and 5%) [18–20]. It should be noted that the secondary cytogenetic abnormality usually described as i(17q) may, in fact, be idic(17p11.2) [19]. Secondary abnormalities are more common in myeloid transformation than in lymphoid. Published data are conflicting but there appears to be little relationship between the nature of secondary cytogenetic events and the lineage most obviously involved at blast transformation. Johansson *et al.* [19] reviewed all published cases and concluded that the only consistent findings were that i(17q), or idic(17p11), was more common in myeloid blast crisis whereas hypodiploidy and −7 were more common in lymphoid blast crisis. However, although balanced translocations show a similar prevalence in myeloid and lymphoid blast crises, the specific balanced translocations associated with myeloid, but not lymphoid, blast crisis include those also seen in AML and MDS, such as t(8;21)(q22;q22), t(15;17)(q22;q12), inv(16)(p13.1q22) and chromosomal rearrangements with a 3q21 or 3q26 breakpoint – t(3;21)(q26.2;q22.1), t(3;3)(q21;q26.2) and inv(3)(q21q26.2) [19]. Chromosome 3q21 and 3q26 abnormalities are often associated with large numbers of lymphocyte-sized micromegakaryocytes [44], whereas i(17q) or idic(17p11) is associated with Pelger–Huët neutrophils and prominent peripheral blood basophilia, the basophil count being usually more than 10% and often more than 20% [19,45]. Acquisition of t(15;17) can be associated with cytological features resembling acute promyelocytic leukaemia and of inv(16) with features resembling M4Eo AML [46]. Recognition of acute promyelocytic transformation may be important because of its responsiveness to all-*trans*-retinoic acid [47]. Monoblastic transformation can occur when there is a rearrangement with an 11q23 breakpoint [48].

In imatinib-treated patients, cytogenetic evolution has been found to be of adverse prognostic significance – but whether or not a cytogenetic response occurs by 3 months is of greater significance [49].

Molecular genetic abnormalities sometimes present include upregulation of *MYC* and *EVI1*, mutation of *RAS* genes, *GATA2* and *RUNX1*, and point mutations and amplification of *BCR-ABL1* [18,50–53]. Amplification of *BCR-ABL1* has been associated with refractoriness to imatinib therapy [52]. Tumour suppressor genes may also be implicated in disease progression. *TP53* abnormalities are common (being seen in about 30% of myeloid blast crises) whereas *RB1* abnormalities are mainly associated with lymphoid blast crises (being seen in about 18% of cases) and possibly megakaryoblastic blast crisis [18,50,51]. Homozygous deletions of *CDKN2A*, encoding $p16^{INK4A}$, are associated with lymphoid blast crises, being seen in about 50% of cases [18,54].

Problems and pitfalls

Beware of missing t(9;22)(q34;q11.2) and *BCR-ABL1* in patients who present either with thrombocytosis or in accelerated phase or blast transformation.

Chronic neutrophilic leukaemia

Chronic neutrophilic leukaemia is a rare Ph-negative condition which, as defined in the WHO classification, is characterized by an increased neutrophil count with only small numbers of circulating granulocyte precursors and no dysplastic features [55]. It appears likely that some cases result from mutation in a multipotent stem cell and others from a mutation in a committed granulocyte precursor [56]. Familial cases have been described [57].

A similar disease phenotype is sometimes seen as an accelerated phase of a myeloproliferative disorder, for example supervening in polycythaemia vera [58].

Clinical and haematological features

Chronic neutrophilic leukaemia occurs predominantly in the middle aged and elderly but has a very wide age range with rare cases having been observed in childhood [59]. Characteristic clinical features are anaemia, splenomegaly and sometimes hepatomegaly. The reported median survival has ranged from little more than a year [59] to 2–3 years [56].

The neutrophil count is markedly increased but the peripheral blood shows few granulocyte precursors (Fig. 5.10). Toxic granulation, Döhle bodies and ring-shaped neutrophil nuclei may be present. Typical myelodysplastic features such as hypogranular neutrophils, the acquired Pelger–Huët anomaly and micromegakaryocytes are not usually seen. A variant has been described in which dysplastic features are prominent [60] but in the WHO classification such cases would not be classified as neutrophilic leukaemia but as MDS/MPN unclassifiable. The NAP score is usually high but may be reduced. Serum vitamin B_{12} concentration is increased. There may be hyperuricaemia, and

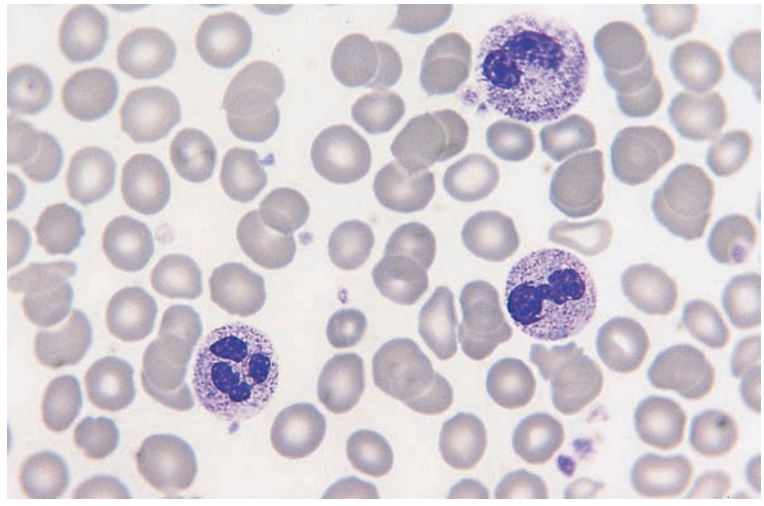


Fig. 5.10 PB film of a patient with neutrophilic leukaemia showing an increase of mature neutrophils, which show toxic granulation. MGG ×100.

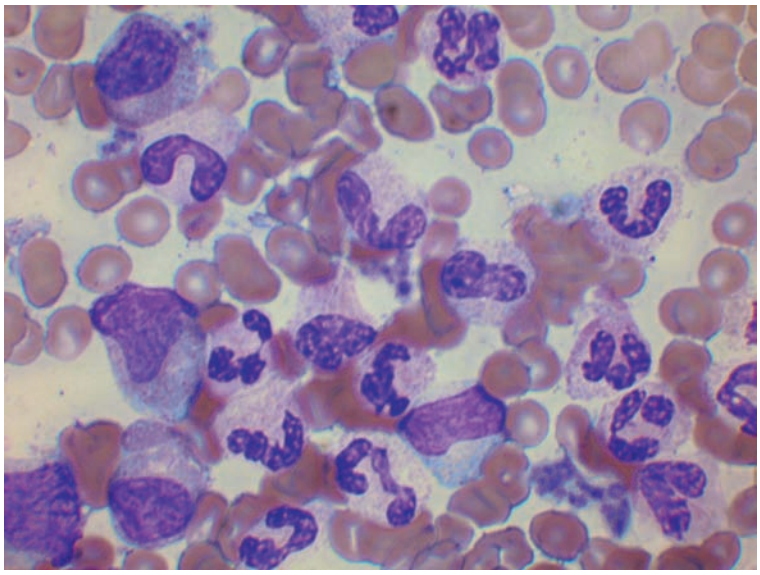


Fig. 5.11 BM film from a patient with chronic neutrophilic leukaemia showing an increase of cells of neutrophil lineage with morphologically normal maturation. MGG ×100.

gout has occurred. Serum granulocyte colony-stimulating factor (G-CSF) is reduced. The bone marrow (Fig. 5.11) is markedly hypercellular with a minor degree of left shift [59]. Some cases have developed bone marrow fibrosis and osteosclerosis [61]; an alternative classification of such cases would be as the hypercellular phase of primary myelofibrosis.

In the WHO classification, the following criteria for the diagnosis of chronic neutrophilic leukaemia are suggested: (i) WBC at least 25×10^9/l, neutrophils and band forms more than 80% of white cells, blast cells less than 1% and immature granulocytes (metamyelocytes to promyelocytes) less than 10% of white cells; (ii) bone marrow showing increased neutrophils and precursors with normal maturation and blast cells less than 5% of nucleated cells; (iii) hepatosplenomegaly; (iv) no identifiable cause of reactive neutrophilia, or, if a potential cause is present (e.g. a non-haematological tumour), demonstration of clonality of myeloid cells; (v) no Ph chromosome or *BCR-ABL1* fusion gene; (vi) no evidence of polycythaemia vera, primary myelofibrosis or essential thrombocythaemia; (vii) no dysplasia; and (viii) monocyte count less than 1×10^9/l [55].

It is important to distinguish chronic neutrophilic leukaemia from the neutrophilic leukaemoid reaction that can occur in association with multiple myeloma and other plasma cell neoplasms in which cytogenetic abnormalities are absent and myeloid cells are considered to be polyclonal [56,62]. However, the report of evolution to acute myelomonocytic leukaemia in only 1.5 years in one such patient is puzzling since the short time interval does not suggest a therapy-related leukaemia and raises the alternative possibility that the patient did actually have chronic neutrophilic leukaemia [63].

Blastic transformation of chronic neutrophilic leukaemia has occurred in a fifth of reported cases [56,64,65]. It may be more frequent in patients with prominent myelodysplastic features (MDS/MPN in the WHO classification) and may be preceded by the development of dysplastic features in those patients who initially lacked dysplasia [55]. In other patients, the disease terminates with a rising white cell count that is refractory to chemotherapy [66].

Cytogenetic and molecular genetic features

By definition, the Ph chromosome and *BCR-ABL1* rearrangement are absent. A number of clonal cytogenetic abnormalities have been reported including trisomy 8, trisomy 9, trisomy 21, del(11q) and del(20q) and other rearrangements involving the long arm of chromosome 20 [56,64,65] but the karyotype is more often normal [19,56]. The *JAK2* V617F mutation has been reported occasionally

Table 5.3 Haematological features of chronic eosinophilic leukaemia and related conditions [2,3].

Syndrome	Usual presentation
Chronic eosinophilic leukaemia, not otherwise specified	As CEL, but acute transformation can subsequently occur
Myeloid and lymphoid neoplasms associated with *PDGFRA* rearrangement	CEL, sometimes with neutrophilia and usually with increased bone marrow mast cells; may undergo T lymphoid or myeloid transformation
Myeloid neoplasms associated with *PDGFRB* rearrangement	CEL, CMML with eosinophilia, aCML with eosinophilia; bone marrow mast cells may be increased; may undergo myeloid transformation
Myeloid and lymphoid neoplasms associated with *FGFR1* rearrangement	CEL, ALL (usually T-lineage, occasionally B-lineage), AML, myeloid or lymphoid transformation following presentation as CEL

aCML, atypical chronic myeloid leukaemia (*BCR-ABL1* negative); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CMML, chronic myelomonocytic leukaemia.

[67]. A patient with t(15;19)(q13;p13.1) was imatinib responsive, suggesting that a trial of this drug may be justified in patients with translocations [68]. In those with an initially normal karyotype, a clonal cytogenetic abnormality may appear during the course of the disease.

Problems and pitfalls

Beware of making a diagnosis of chronic neutrophilic leukaemia in patients with plasma cell neoplasms. Serum protein electrophoresis is indicated in all patients who lack definitive evidence of a myeloid neoplasm.

Chronic eosinophilic leukaemia

Cases of leukaemia with eosinophilic differentiation and with 20% or more blasts in the bone marrow are regarded, in the WHO classification, as acute leukaemia. Eosinophil proliferation is also sometimes associated with ALL and in these cases the eosinophilia is usually reactive. Cases of leukaemia in which eosinophils predominate or are prominent but with bone marrow blast cells being fewer than 20% are designated chronic eosinophilic leukaemia (CEL), unless they have a cytogenetic abnormality, such as t(8;21)(q22;q22), that identifies them as AML. In the 2008 WHO classification, cases characterized by rearrangement of one of three tyrosine kinase genes (*PDGFRA*, *PDGFRB* or *FGFR1*) are classified according to the molecular abnormality while other cases are designated chronic eosinophilic leukaemia, not otherwise specified (CEL, NOS) [2,3] (Table 5.3). Clinical and pathological features are similar in these different groups but there are some disease characteristics associated more specifically with specific syndromes (see below).

Clinical and haematological features

Patients with eosinophilic leukaemia usually present with anaemia, thrombocytopenia, hepatomegaly, splenomegaly and signs and symptoms of damage to the heart and other tissues caused by release of eosinophil granule contents. Lymphadenopathy can occur. Survival is variable but has often been quite short, as a consequence either of disease progression or of organ damage. With the discovery that myeloid neoplasms resulting from rearrangement of *PDGFRA or PDGFRB* are sensitive to imatinib, a much better survival may be anticipated in this subset of patients.

Eosinophils are increased in the blood and there may also be an increase of eosinophil precursors including blast cells (Fig. 5.12). An eosinophil count of at least 1.5×10^9/l is a WHO criterion for the diagnosis of CEL, NOS [3] but in many patients with CEL the count is much higher. Eosinophils often show morphological abnormalities such as vacuolation, degranulation, hypolobulation and hyperlobulation but it should be noted that such abnormalities can also be seen in reactive eosinophilia. Some patients also have neutrophilia or monocytosis, with or without circulating granulocyte precursors. Eosinophil granules were reported, in one patient, to show weak to moderate chloroacetate esterase (CAE) activity but whether this is common is not known [69]; the same patient had

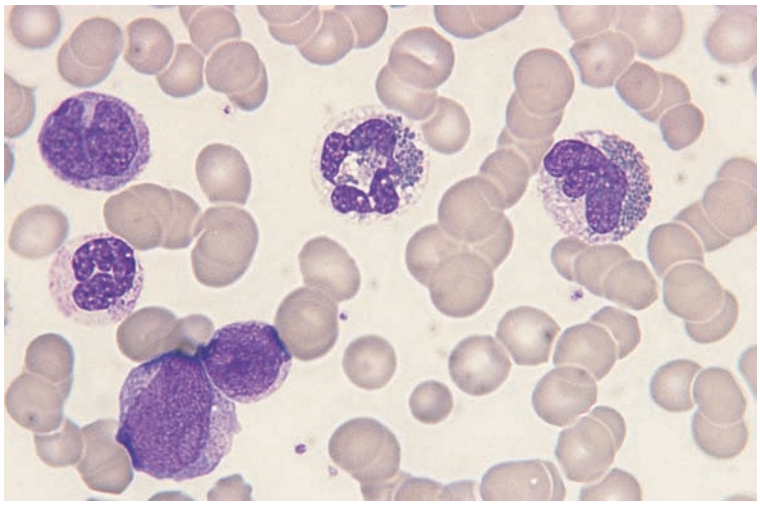


Fig. 5.12 PB film of a patient with chronic eosinophilic leukaemia with trisomy 10 showing a neutrophil, two abnormal eosinophils, a granulocyte precursor and two blast cells. MGG ×100.

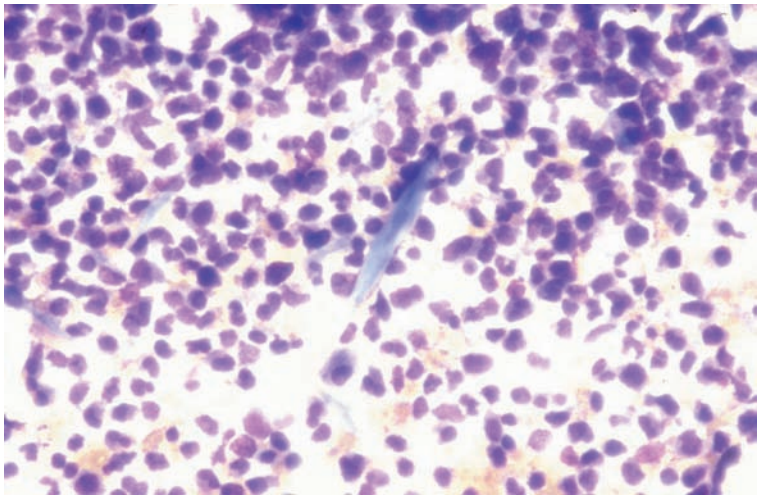


Fig. 5.13 BM film from a patient with eosinophilic leukaemia showing a Charcot–Leyden crystal. MGG ×40.

periodic acid–Schiff-positive cytoplasm [69]. One patient has been described in whom there was cycling of the WBC, Hb and platelet count [70].

The bone marrow shows increased eosinophils and precursors and sometimes also an increase in neutrophils, monocytes and their precursors, or blast cells. Occasionally Charcot–Leyden crystals are present (Fig. 5.13). Trephine biopsy sections show the same features and, in addition, in patients with disease related to rearrangement of *PDGFRA* or *PDGFRB*, may show increased mast cells.

A particular problem occurs in establishing the diagnosis of eosinophilic leukaemia in patients with a marked increase of mature eosinophils but with no excess of blast cells. The differential diagnosis is between reactive eosinophilia, eosinophilic leukaemia and the idiopathic hypereosinophilic syndrome. The nature of the latter condition is not certain. Although some patients can be recognized in retrospect as having had eosinophilic leukaemia, others die as a result of eosinophil-mediated tissue damage without any incontrovertible evidence of a leukaemic process having emerged. Marked eosinophilia and the presence of morphological abnormalities in eosinophils are of little use in confirming the diagnosis of leukaemia since they

may occur also in reactive eosinophilia. The demonstration of a clonal cytogenetic or molecular genetic abnormality confirms a diagnosis of CEL as does a peripheral blood blast cell count of more than 2% or a bone marrow blast count of 6–19% [3]. If these criteria are not met and if no other cause of the eosinophilia can be found, the non-committal diagnosis of idiopathic hypereosinophilic syndrome may be made [71]. This diagnosis has become much less common since the discovery that many such cases actually have CEL as a result of a cryptic chromosomal deletion leading to formation of a *FIP1L1-PDGFRA* fusion gene [72].

In the past some cases classified as idiopathic hypereosinophilic syndrome subsequently developed a granulocytic sarcoma or transformation to AML, providing evidence that the disorder was leukaemic/myeloproliferative from the outset. Whether this will continue to happen if CEL related to *PDGFRA* rearrangement is excluded at presentation remains to be seen.

Cytogenetic and molecular genetic features

Recurrent genetic abnormalities that define specific WHO categories of myeloid or lymphoid and myeloid neoplasms with eosinophilia include: (i) t(5;12)(q31~33;p12) and other translocations with *PDGFRB* rearrangement; (ii) *FIP1L1-PDGFRA* fusion and other rearrangements of *PDGFRA*; and (iii) t(8;13)(p11;q12) and other rearrangements of *FGFR1*. These three groups of disorders are discussed below.

A number of clonal cytogenetic abnormalities have been observed in CEL, NOS, including monosomy 7, trisomy 8, i(17q), trisomy 15, del(20)(q11q12) and the presence of trisomy 8 and trisomy 21 in different clones. A number of cases have been reported with t(8;9)(p21-23;p23-24) and a *PCM1-JAK2* fusion gene [73]. A single patient with CEL has been reported with an *ETV6-ABL1* fusion gene associated with a complex chromosomal rearrangement involving chromosomes 9, 12, and 17 [74], the same fusion gene resulting from t(9;12)(q34;p13) having been associated with AML with eosinophilia. Disease associated with *ETV6-ABL1* is sensitive to imatinib and nilotinib [75]. A further patient with CEL had an *ETV6-FLT3* fusion gene associated with t(12;13)(p13.1;q12.3–13) [76] and another with an MPN with eosinophilia had *ETV6-SYK* fusion associated with t(9;12)(q22;p12) [77]. *JAK2* V617F has been reported occasionally and may be associated with female preponderance and cardiac damage [78]; in one patient transformation to AML occurred [78].

An increase in expression of *WT1*, detected by RQ-PCR, may be useful in distinguishing eosinophilic leukaemia and the idiopathic hypereosinophilic syndrome (increased expression) from reactive eosinophilia (normal expression) [79].

Problems and pitfalls

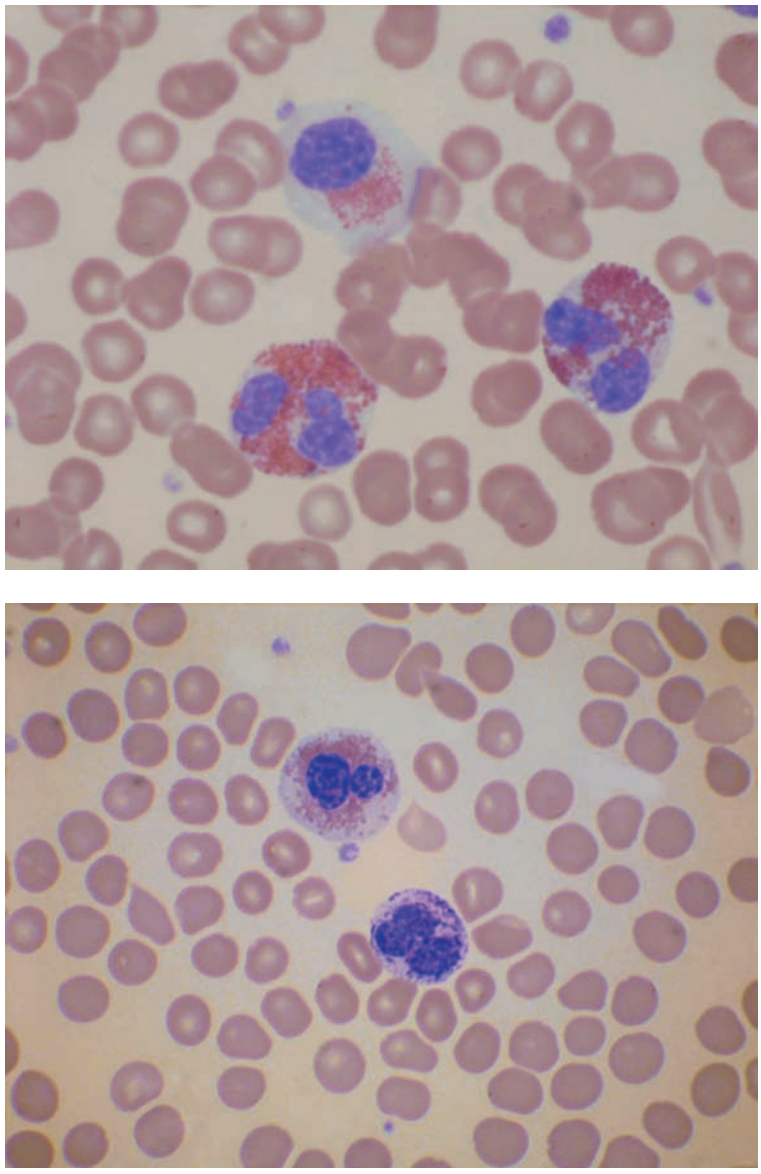
It is important to be aware that there may be some patients with chronic eosinophilic leukaemia whose disease cannot be recognized because they do not have an increase of blast cells, a clonal cytogenetic abnormality or a known molecular genetic abnormality.

Myeloid and lymphoid neoplasms associated with *FIP1L1-PDGFRA* fusion

The discovery of a recurring molecular genetic abnormality in a group of patients with idiopathic hypereosinophilic syndrome led to the recognition of a new category of chronic eosinophilic leukaemia with normal cytogenetic analysis and a fusion gene resulting from a cryptic deletion [72,80]. Subsequently it was recognized that T lymphoblastic and myeloid transformations occur and that in some patients the disease presents as acute leukaemia [81]. The causative mutation arises in a pluripotent stem cell able to give rise to eosinophils, neutrophils, monocytes, B lymphocytes, T lymphocytes and mast cells [82] but in chronic phase disease abnormal proliferation is largely confined to eosinophils, neutrophils and mast cells. Because of its responsiveness to imatinib, it is important that this syndrome is recognized.

Clinical and haematological features

Patients are almost always male. The median age of presentation is in early middle age but the range is wide, extending from childhood to old age. There is often splenomegaly, less often hepatomegaly, and damage to the heart and other organs is frequent. There may be mild anaemia and thrombocytopenia, a moderately elevated WBC and a moderately to



(a)

(b)

Fig. 5.14 PB film in a patient with *FIP1L1-PDGFRA* showing: (a) three eosinophils, one of which has a non-lobulated nucleus and is extensively degranulated; (b) one eosinophil, which is partly degranulated, and a granulocyte with abnormal tinctorial qualities (such granulocytes, of uncertain lineage, may be seen in patients with hypereosinophilia). MGG ×100.

markedly elevated eosinophil count (Fig. 5.14). Reported cases have had eosinophil counts as low as 0.9×10^9/l but it is not clear if low counts were the result of corticosteroid or other therapy [83]. The neutrophil count may be increased and some circulating granulocyte precursors may be present. Serum vitamin B_{12} is markedly elevated. Serum tryptase is elevated but to a lesser extent than is seen is systemic mastocytosis [80]. Serum immunoglobulin E is elevated in only a minority of patients [83]. The bone marrow is hypercellular with increased eosinophils and precursors (Fig. 5.15). Trephine biopsy shows an increase of eosinophils and precursors (Fig. 5.16) and, in addition, mast cells are often increased and may be atypical, e.g. spindle shaped; usually they form loose clusters [80] but sometimes there are cohesive aggregates or sheets, the features thus resembling systemic

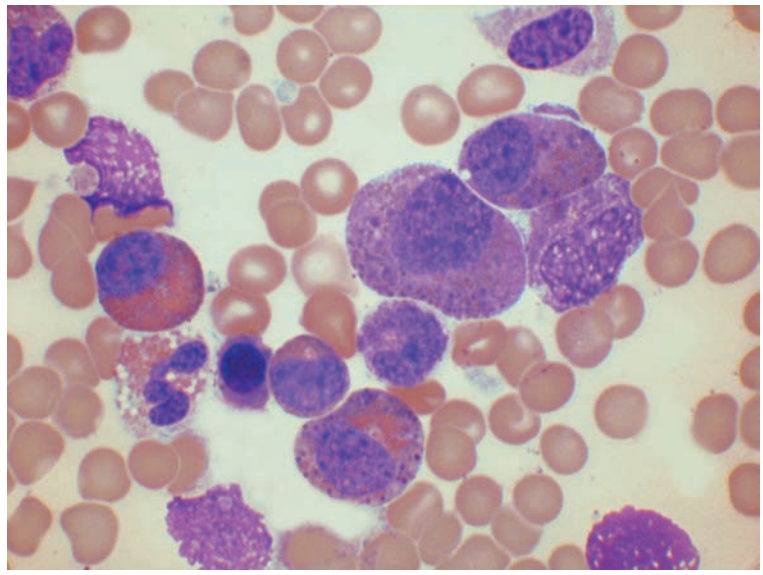


Fig. 5.15 BM film in a patient with *FIP1L1-PDGFRA* showing eosinophil precursors, some of which have pro-eosinophilic granules with basophilic staining characteristics; there is also a vacuolated, partly degranulated, mature eosinophil. MGG ×100.

mastocytosis. The mast cells, like those of systemic mastocytosis, often show aberrant expression of CD25 but they differ in that CD2 is not usually aberrantly expressed [80]. Reticulin deposition is often increased.

Transformation to acute leukaemia can occur after a variable period of time [81]. This may be acute eosinophilic leukaemia but is equally likely to be FAB subtypes M0, M1, M2 or M4 and is sometimes T lymphoblastic leukaemia [81].

Cytogenetic and molecular genetic features

This condition is defined by the presence of a *FIP1L1-PDGFRA* fusion gene resulting from an interstitial deletion at 4q12 [72] or by other rearrangement of *PDGFRA* [2] (Table 5.4). In patients with *FIP1L1-PDGFRA* fusion, cytogenetic analysis may be normal or show a clonal cytogenetic abnormality that is related or unrelated to the underlying molecular abnormality. Related abnormalities are translocations with a 4q12 breakpoint such as t(1;4)(q44;q12)

Table 5.4 Chronic eosinophilic leukaemia and related conditions associated with rearrangement of *PDGFRA* [2].

Chromosomal abnormality	Fusion gene	Number of cases	Haematological manifestations
Usually normal with cryptic deletion at 4q12; occasionally translocation with 4q12 breakpoint	*FIP1L1-PDGFRA*	The great majority of cases	CEL, often with neutrophilia and abnormal bone marrow mast cells; T lymphoid and acute myeloid transformations can occur; AML with eosinophilia
t(4;22)(q12;q11)	*BCR-PDGFRA*	5	Intermediate features between CEL and Ph-positive CGL; T and B lymphoid transformation can occur
Complex involving chromosomes 3, 4, 10 and probably 13	*KIF5B-PDGFRA*	1	CEL
ins(9;4)(q33;q12q25)	*CDK5RAP2-PDGFRA*	1	CEL
t(2;4)(p24;q12)	*STRN-PDGFRA*	1	CEL
t(4;12)(q12;p13)	*ETV6-PDGFRA*	1	CEL

AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CGL, chronic granulocytic leukaemia.

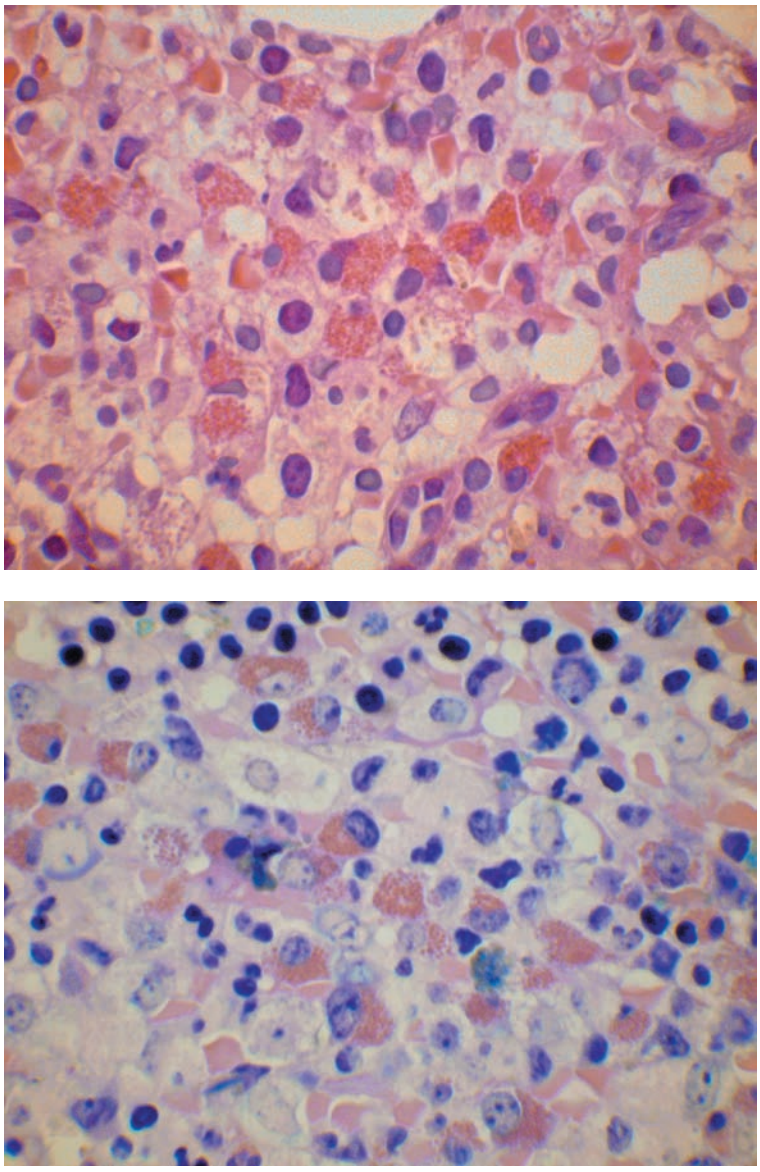


Fig. 5.16 Trephine biopsy sections from a patient with a *FIP1L1-PDGFRA*-associated myeloproliferative neoplasm (MPN) showing an increase of eosinophils and precursors: (a) haematoxylin and eosin (H&E) ×100; (b) Giemsa ×100.

[72] or t(4;10)(q12;p11) [84]. Unrelated abnormalities have included trisomy 8 [85] and a complex karyotype including trisomy 8 and trisomy 19 [72]. Additional cytogenetic abnormalities, such as trisomy 8 and trisomy 9, may be present at evolution to AML [81]. Acquired resistance to imatinib as a result of a further mutation (e.g. T674I or D842V) in the fusion gene has been described in several patients [86,87].

Variant translocations associated with rearrangement of *PDGFRA* and a different partner gene are summarized in Table 5.4. The associated syndrome is clinically and haematologically similar to that associated with *FIP1L1-PDGFRA* fusion, with the exception that haematological features associated with *BCR-PDGFRA* fusion are often closer to those of CGL.

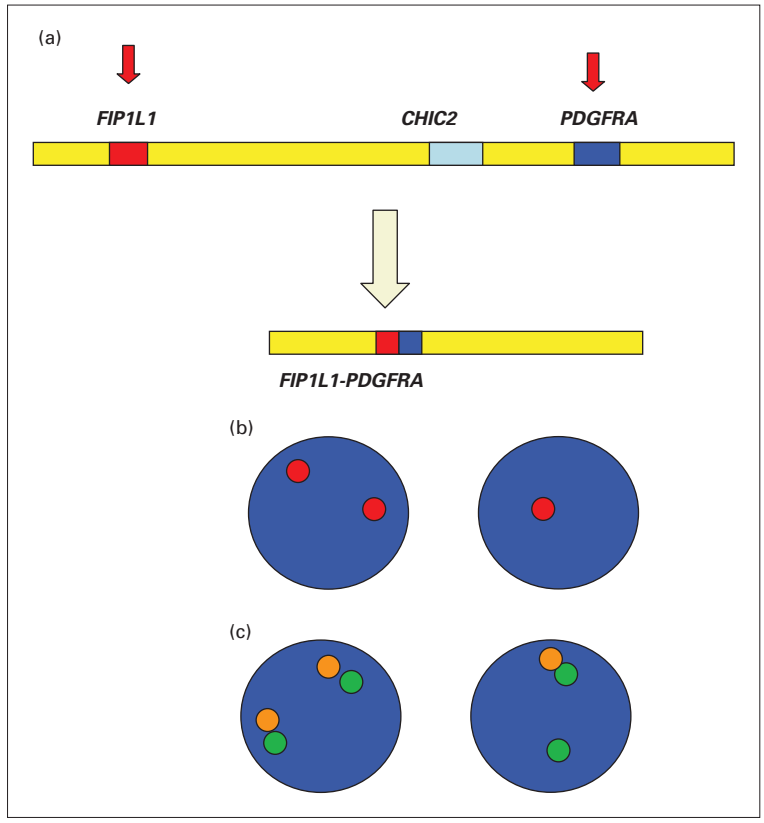


Fig. 5.17 Diagrams illustrating the loss of *CHIC2* when a *FIP1L1-PDGFRA* fusion gene is formed as a result of a cryptic deletion at 4q12: (a) diagram showing the occurrence of an interstitial deletion with loss of *CHIC2*; the red arrows indicate the two breakpoints; (b) a FISH technique using a *CHIC2* probe; a normal cell (left) has two signals whereas a leukaemic cell (right) has a single signal; (c) an alternative FISH technique using a *CHIC2* probe (orange) and a probe for the *FGFR3* gene at 4p16.3 (green); the normal cell (left) has two pairs of juxtaposed orange and green signals while the leukaemic cell has one normal pair of signals and a single green *FGFR3* gene, the *CHIC2* gene from that chromosome having been lost.

Problems and pitfalls

Because very effective treatment is available it is crucial not to miss this diagnosis. PCR may be insufficient. Nested PCR and FISH (Fig. 5.17) are recommended. Because of the potential for cardiac damage, delay in diagnosis should also be avoided. It is important to be aware that patients who present with either AML with eosinophilia or T lymphoblastic leukaemia/lymphoma with eosinophilia should be investigated for a *FIP1L1-PDGFRA* fusion gene since patients with this fusion gene may respond well to tyrosine kinase inhibitors, even when they present with the disease in the acute phase.

Myeloid neoplasms associated with rearrangement of *PDGFRB*

Myeloid neoplasms associated with rearrangement of *PDGFRB* form a somewhat heterogeneous group. The majority of patients have a chronic myeloid neoplasm with prominent eosinophilia, t(5;12)(q31~q33;p12) and an *ETV6-PDGFRB* fusion gene but at least 21 fusion partners have been reported (Table 5.5). This condition is imatinib responsive. To date all neoplasms reported have been myeloid.

Clinical and haematological features

This group of disorders is twice as common in men as in women. The age of presentation is very variable with the peak incidence in early middle age. Clinical features include splenomegaly and, sometimes, cardiac damage or skin infiltration. The haematological features are variable. In addition to the frequent occurrence of eosinophilia (Figs 5.18 and 5.19), some patients have had neutrophilia, monocytosis, basophilia or circulating granulocyte precursors. There may be anaemia, thrombocytopenia and multilineage myelodysplasia. Some cases

Table 5.5 Chronic eosinophilic leukaemia and related conditions associated with rearrangement of *PDGFRB* [2,71,88–96]. (Modified from reference 2.)

Chromosomal rearrangement	Fusion gene*	Haematological presentation
t(5;12)(q31~q33;p12) or variant	*ETV6-PDGFRB*	CEL or other MPN (CML, primary myelofibrosis transforming to AML) or MDS/MPN (CMML, aCML), usually with eosinophilia; bone marrow mast cells may be increased; AML
t(1;3;5)(p36;p21;q33)	*WDR48-PDGFRB* (*WDR48* is at 3p22)	CEL
der(1)t(1;5)(p34;q33), der(5)t(1;5)(p34;q15), der(11)ins(11;5)(p12;q15q33)	*GPIAP1-PDGRFB* (*GPIAP1* is at 11p13)	CEL
t(1;5)(q21;q33)	*TPM3-PDGFRB*	CEL
t(1;5)(q23;q33)	*PDE4DIP-PDGFRB*	MDS/MPN with eosinophilia
t(2;5)(p21;q33)	*SPTBN1-PDGFRB*	MPN with eosinophilia
t(3;5)(p21-25;q31-35)	*GOLGA4-PDGFRB*	CEL
t(4;5;5)(q23;q31;q33), t(4;5)(q21.2;q31.3) or t(4;4)(q21;q33)	*PRKG2-PDGFRB*	Two cases with chronic basophilic leukaemia and abnormal bone marrow mast cells [91,92], one case with MPN with abnormal mast cells and an eosinophil count of 1.1×10^9/l [93]
t(5;7)(q33;q11.2)	*HIP1-PDGFRB*	CMML with eosinophilia
t(5;10)(q33;q21)	*CCDC6-PDGFRB*	MPN with eosinophilia or aCML
t(5;12)(q31-33;q24)	*GIT2-PDGFRB*	CEL
t(5;12)(q33;p13.3)	*ERC1-PDGFRB*	AML (without eosinophilia) [94]
t(5;14)(q33;q24)	*NIN-PDGFRB*	Ph-negative CML (13% eosinophils)
t(5;14)(q33;q32)	*TRIP11-PDGFRB*	Occurred at relapse of AML, associated with appearance of eosinophilia [95]
t(5;14)(q33;q32)	*CCDC88C*(*KIAA1509*)-*PDGFRB*	CMML with eosinophilia
t(5;15)(q33;q22)	*TP53BP1-PDGFRB*	Ph-negative CML with prominent eosinophilia
t(5;16)(q33;p13)	*NDE1-PDGFRB*	CMML with eosinophilia
t(5;17)(q33;p11.2)	*SPECC1-PDGFRB*	JMML with eosinophilia
t(5;17)(q33;p13)	*RABEP1-PDGFRB*	CMML (without eosinophilia)
t(5;17)(q33-34;q11.2)	*MYO18A-PDGFRB*	MPN with eosinophilia [96]

aCML, atypical chronic myeloid leukaemia; AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CML, chronic myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; JMML, juvenile myelomonocytic leukaemia; MDS/MPN, myelodysplastic/myeloproliferative neoplasm; MPN, myeloproliferative neoplasm.
* *PDGFRB* was also found to be rearranged but without the partner gene being determined in t(5;12)(q33;q13) [90].

are best characterized as CEL but others as either atypical chronic myeloid leukaemia (aCML) with eosinophilia or CMML with eosinophilia (Table 5.5). Although eosinophilia is very common, it does not appear to be invariable. In two patients with t(4;5)(q21.2;q31.3) or a variant translocation and a *PRKG2-PDGFRB* fusion gene, the haematological features were those of chronic basophilic leukaemia [91,92]. Bone marrow mast cells may be increased and may show aberrant expression of CD25; serum tryptase may be increased [91,92]. Serum vitamin B_{12} concentration is often increased [83]. Acute transformation occurred in 16% of reported patients [89]. The median survival of all patients prior to the availability of imatinib was less than 2 years [89].

Cytogenetic and molecular genetic features

This condition is defined by the presence of rearrangement of *PDGFRB* in the context of a haematological neoplasm. The most frequent cytogenetic abnormality is t(5;12)(q31~q33;p12) (Fig. 5.20). The 5q breakpoint appears variable because these

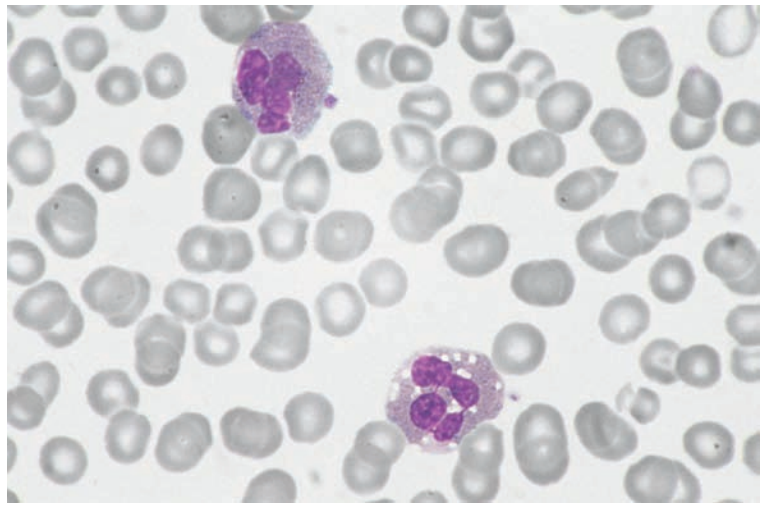


Fig. 5.18 PB film from a patient with chronic eosinophilic leukaemia with t(5;12)(q33;p13). One of the eosinophils is vacuolated but overall cytological abnormalities were fairly minor. (By courtesy of Dr Elisa Granjo, Porto.)

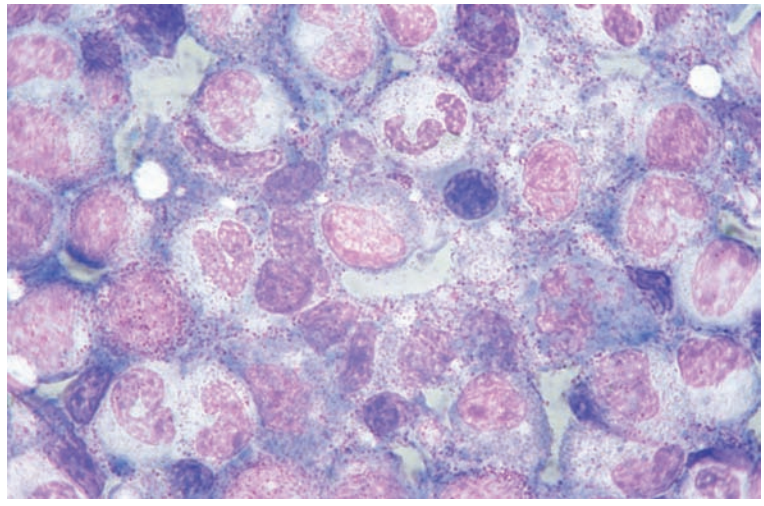


Fig. 5.19 BM film from a patient with chronic eosinophilic leukaemia with t(5;12)(q33;p13) showing a marked increase in eosinophils and precursors (same patient as Fig. 5.18).

rearrangements are often complex and FISH analysis may be misleading [89]. Other reported chromosomal abnormalities are summarized in Table 5.5. The eosinophils are part of the leukaemic clone [97]. As in the case of CGL, development of a clonal cytogenetic abnormality (trisomy 8) during imatinib therapy in cells that were not part of the leukaemic clone has been reported [98].

Problems and pitfalls

Because very effective treatment is available it is important to recognize this syndrome. The 5q breakpoints are variable. If confirmatory molecular analysis is not available a trial of imatinib is justified in patients with breakpoints in this region.

Myeloid and lymphoid neoplasms associated with rearrangement of *FGFR1*

Haematological neoplasms associated with rearrangement of *FGFR1* at 8p11, previously referred to as the 8p11 syndrome, form a heterogeneous group

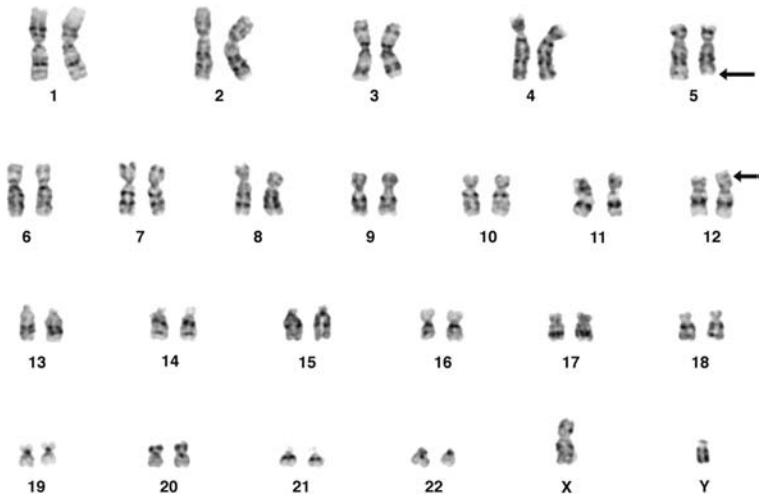


Fig. 5.20 Karyogram of a patient with chronic eosinophilic leukaemia with t(5;12)(q33;p13) (same patient as Figs 5.18 and 5.19). (By courtesy of Sofia Dória, Porto.)

Table 5.6 Lymphoid and myeloid neoplasms associated with rearrangement of *FGFR1** [2,71,99–101]. (Modified from reference 2.)

Cytogenetics	Molecular genetics	Number†	Haematological syndromes
t(8;13)(p11;q12)	*ZNF198-FGFR1*	21	CEL, T-ALL/T-LBL, AML, B-lineage ALL
t(8;9)(p11;q33)	*CEP110-FGFR1*	8	CEL, T-ALL/T-LBL; sometimes there is monocytosis or thrombocytosis
t(6;8)(q27;p11-12)	*FGFR1OP1-FGFR1*	6	CEL, T-ALL, AML, B-lineage ALL, polycythaemia in four patients
t(8;22)(p11;q11)	*BCR-FGFR1*	5	CML; myeloid transformation may occur; one T + B lymphoid transformation
t(7;8)(q34;p11)	*TRIM24-FGFR1*	1	AML with eosinophilia
t(8;17)(p11;q23)	*MYO18A-FGFR1*	1	CML with severe thrombocytopenia
t(8;19)(p12;q13.3)	*HERVK-FGFR1*	1	AML with 45% bone marrow eosinophils
ins(12;8)(p11;p11p22)	*FGFR1OP2-FGFR1*	1	T-LBL and mild eosinophilia that progressed rapidly to AML

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CML, chronic myeloid leukaemia; T-ALL, T-lineage acute lymphoblastic leukaemia; T-LBL, T-lineage lymphoblastic lymphoma.
* In addition, *FGFR1* rearrangement has been found in association with t(8;12)(p11;q15) associated with T lymphoblastic lymphoma and MPN and with t(8;17)(p11;q25) associated with CML and systemic mastocytosis but the suspected involvement of *FGFR1* in t(8;11)(p11;p15) was not confirmed.
† Numbers updated to 2008 from reference 101.

of lymphoid and myeloid neoplasms with prominent eosinophilia (Table 5.6). There is a slight male preponderance and a relatively young age of presentation (median age of reported patients is 32 years) [101]. Presenting disease features include splenomegaly and lymphadenopathy, the latter either due to infiltration by myeloid cells or to blastic transformation. The prognosis is poor although some patients have survived following bone marrow transplantation.

Clinical and haematological features

Presentation is most often with haematological features of an MPN with eosinophilia (Figs 5.21 and 5.22),

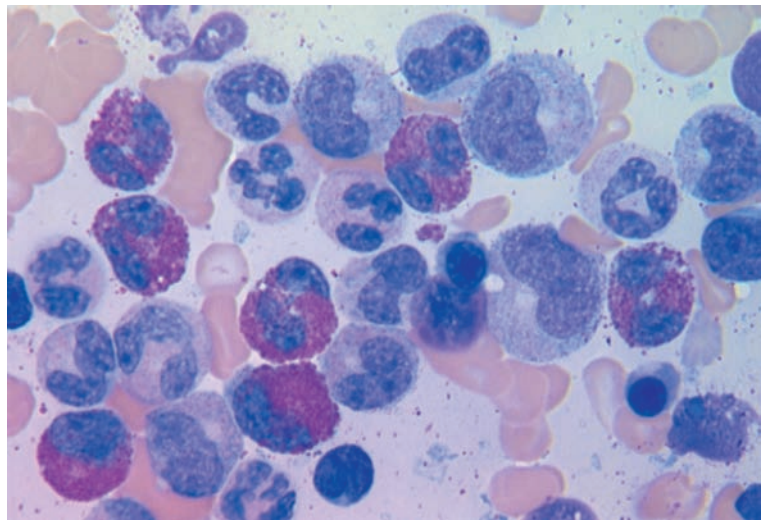


Fig. 5.21 BM film from a 27-year-old man with a myeloid and lymphoid neoplasm (chronic eosinophilic leukaemia and T lymphoblastic lymphoma) associated with t(8;13)(p11;q12) showing an increase of neutrophils, neutrophil precursors, eosinophils and eosinophil precursors. H&E ×100. (By courtesy of Dr Colm Keane, Princess Alexandra Hospital, Brisbane.)

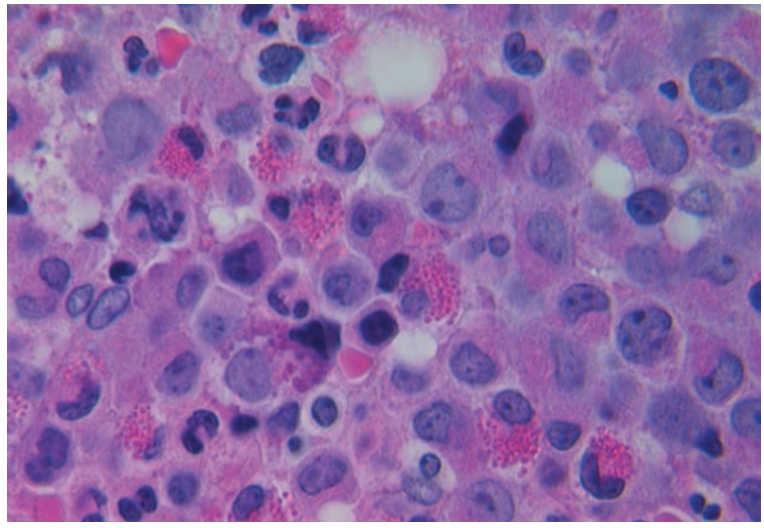


Fig. 5.22 BM trephine biopsy section from a patient with a myeloid and lymphoid neoplasm associated with t(8;13)(p11;q12) (same patient as Fig. 5.21) showing hypercellularity and an increase in eosinophils and precursors. H&E ×100. (By courtesy of Dr Colm Keane.)

with or without simultaneous T lymphoblastic leukaemia/lymphoma (Fig. 5.23). Patients who present in chronic phase usually subsequently suffer myeloid transformation (myeloid sarcoma or AML) or lymphoid transformation (mainly T lymphoblastic leukaemia/lymphoma but sometimes B lymphoblastic leukaemia/lymphoma). Occasionally a patient suffers both a lymphoid and a myeloid transformation [102–104]. T lymphoblasts, B lymphoblasts and myeloid cells belong to the cytogenetically abnormal neoplastic clone. Four patients have been reported with polycythaemia as part of the syndrome, all associated with t(6;8)(q27;p11-12) and in one case with a coexisting *JAK2* V617F mutation [105].

Cytogenetic and molecular genetic features

The majority of cases have been associated with t(8;13)(p11;q12) (Figs 5.24 and 5.25), t(8;9)(p11;q33) or t(6;8)(q27;p11-12). Other translocations and fusion genes that may underlie this syndrome are shown in Table 5.6.

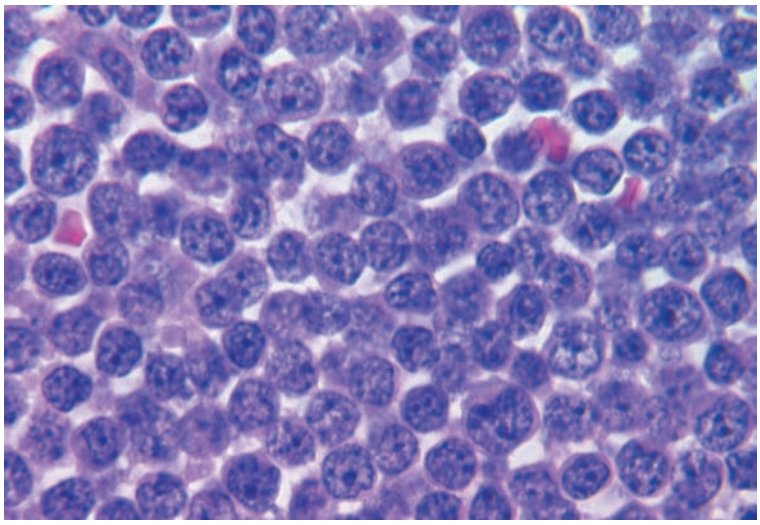


Fig. 5.23 Lymph node biopsy from a patient with a myeloid and lymphoid neoplasm associated with t(8;13)(p11;q12) showing lymphoblastic infiltration of a lymph node (same patient as Figs 5.21 and 5.22). H&E ×100. (By courtesy of Dr Colm Keane.)

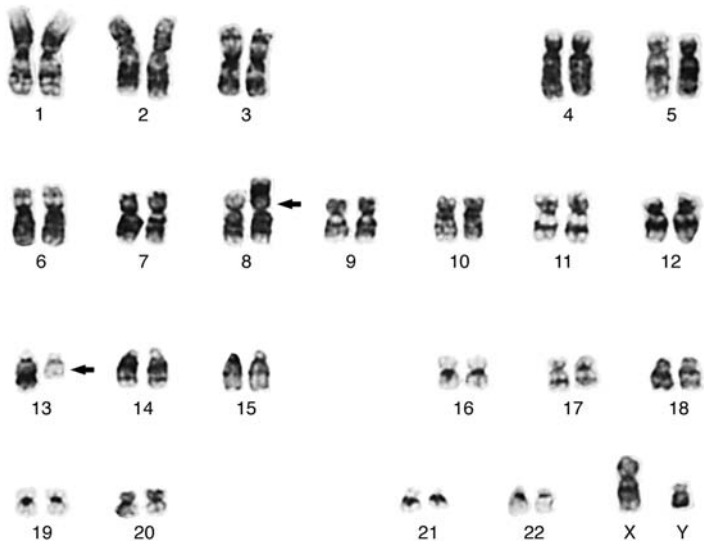


Fig. 5.24 Karyogram showing t(8;13)(p11;q12). (By courtesy of Jill Elliott and the Regional Cytogenetics Service, Sheffield Children's Hospital.)

Problems and pitfalls

Because of its poor prognosis and the current lack of any specific targeted drug therapy, stem cell transplantation should be considered. Recognition of this syndrome is therefore important.

Chronic basophilic leukaemia

Cases of leukaemia showing basophilic differentiation with fewer than 20% blast cells in the peripheral blood and bone marrow and without a *BCR-ABL1* fusion gene [106] are classified as chronic basophilic leukaemia. The rare patients whose neoplastic cells have *PDGFRB* rearrangement are also excluded from this category.

Clinical and haematological features

Clinical feature are splenomegaly and sometimes signs and symptoms consequent on histamine excess [107]. The peripheral blood and bone marrow show dominant basophilic differentiation.

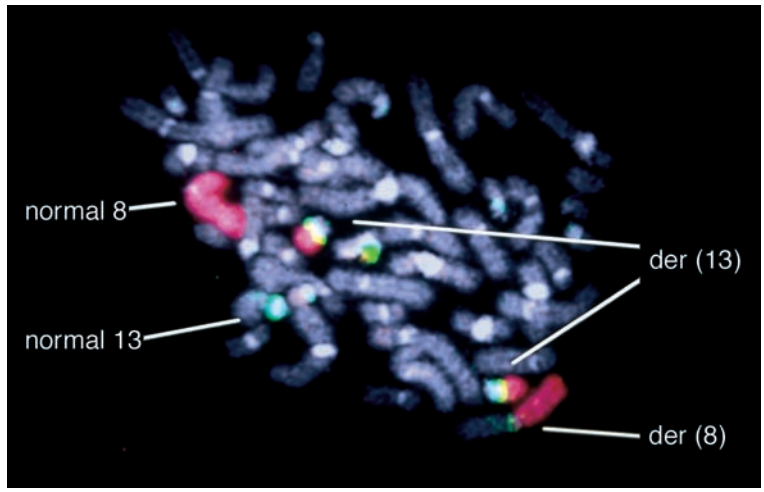


Fig. 5.25 FISH analysis from a patient with an MPN associated with t(8;13)(p11;q12); in this patient there was subsequently transformation to acute myeloid leukaemia. (By courtesy of Dr Donald Macdonald and Dr A. Chase, Hammersmith Hospital.)

Cytogenetic and molecular genetic features
By definition, cases are Ph negative and do not have *BCR-ABL1* fusion.

Atypical (Ph-negative) chronic myeloid leukaemia, *BCR-ABL1* negative

Atypical chronic myeloid leukaemia differs from CGL clinically, haematologically and genetically. In the WHO classification it falls into the group of MDS/MPN. The WHO diagnostic criteria are summarized in Table 5.7 [108].

Clinical and haematological features
This condition is much less common than CGL. There is a wide age range but patients tend to be elderly, on average 15–20 years older than those with CGL. Most patients present with splenomegaly, symptoms of anaemia or, less often, thrombocytopenia

Table 5.7 WHO diagnostic criteria for atypical chronic myeloid leukaemia, *BCR-ABL1* negative, and chronic myelomonocytic leukaemia [108,113].

	Atypical chronic myeloid leukaemia	Chronic myelomonocytic leukaemia
Peripheral blood	WBC at least 13×10^9/l	WBC ranges from normal (or slightly reduced) to high
	Neutrophil precursors at least 10%	Neutrophil precursors usually less than 10%
	Basophils usually less than 2%	Mild or no basophilia
	Monocytes less than 10% of leucocytes	Monocytes more than 1×10^9/l and almost always more than 10%
	Less than 20% blasts (plus promonocytes)	Less than 20% blasts (plus promonocytes)
Bone marrow	Hypercellular, granulocytic dysplasia Less than 20% blasts (plus promonocytes)	Hypercellular, less than 20% blasts (plus promonocytes)
Peripheral blood or bone marrow	Granulocytic dysplasia	Dysplasia in one or more lineages; if dysplasia absent, clonal abnormality present or monocytosis persists for at least 3 months and no other cause found
Cytogenetics/genetics	Absence of t(9;22)(q34;q12.2) and *BCR-ABL1* Absence of rearrangement of *PDGFRA*, *PDGFRB*, *FGFR1*	

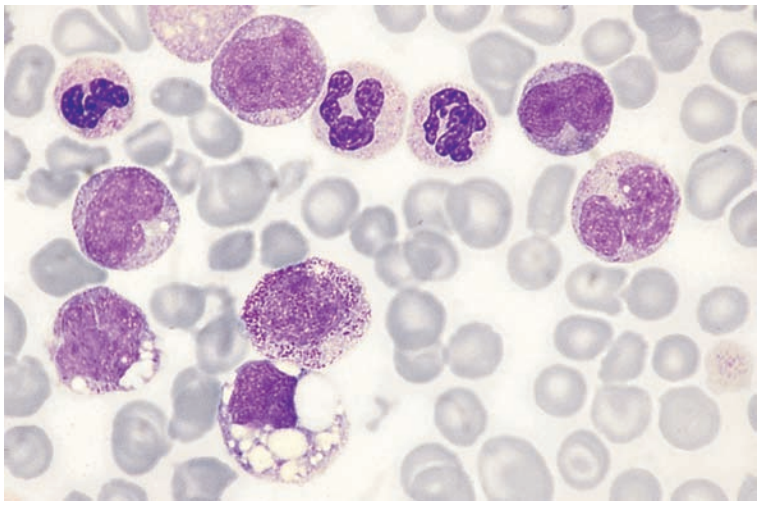


Fig. 5.26 PB film from a patient with chronic myeloid leukaemia supervening in primary myelofibrosis. MGG ×100.

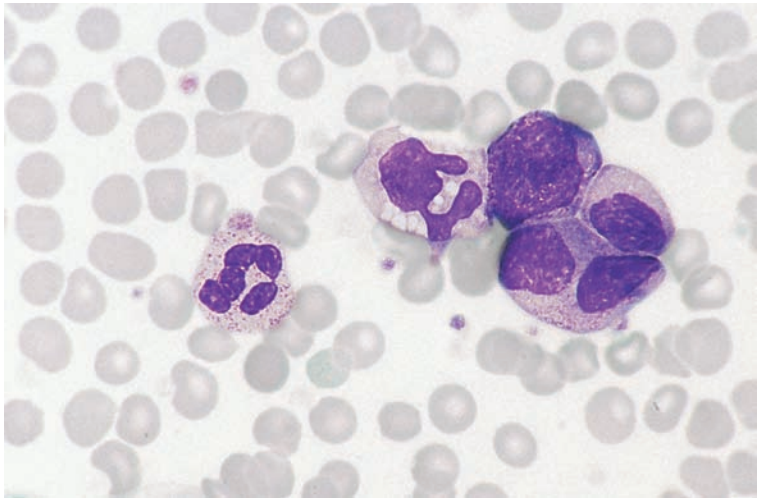


Fig. 5.27 PB film of a patient with atypical (Ph-negative) chronic myeloid leukaemia (aCML) showing a neutrophil, a monocyte, a promyelocyte and two myelocytes, one of which is binucleate. MGG ×100.

and the haematological features of a chronic myeloid leukaemia. Others initially present with MDS, mainly refractory anaemia, and subsequently develop the features of aCML [109]. Similar haematological features can also develop in the terminal phase of polycythaemia vera, essential thrombocythaemia and primary myelofibrosis (Fig. 5.26). Prognosis of aCML is poor with death resulting from bone marrow failure or transformation to AML.

The peripheral blood shows leucocytosis (Fig. 5.27) with an increase of both neutrophils and their precursors but, in comparison with CGL, monocytosis is usually more prominent while eosinophilia and basophilia are less common [110]. Monocytosis is less prominent than in CMML. On average, the WBC is lower than in CGL whereas anaemia and thrombocytopenia are more common. Dysplastic features are common. Neutrophils may be hypogranular or have nuclei showing an acquired Pelger–Huët anomaly or exaggerated clumping of chromatin (Fig. 5.28). Granulocyte precursors are usually 10–20% of circulating white cells and they are also sometimes hypogranular. Blast cells plus promonocytes are usually less than 5% and,

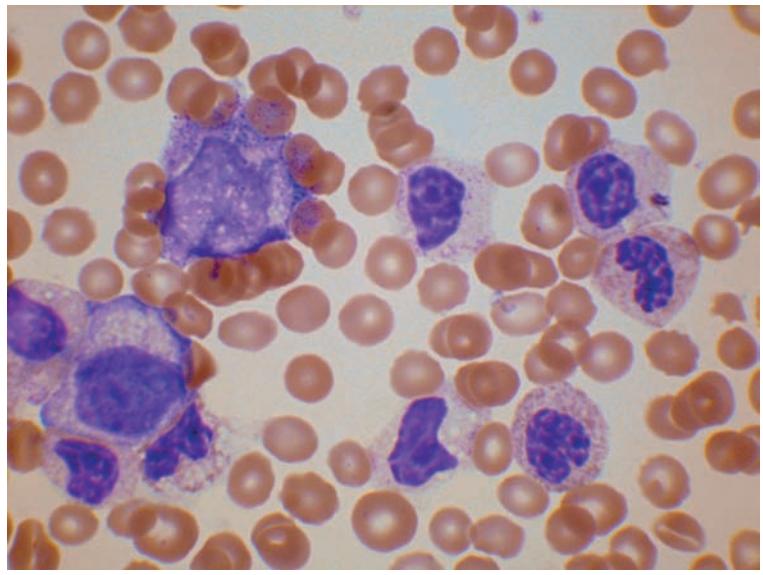


Fig. 5.28 PB film of a patient with aCML showing abnormal chromatin clumping in cells of neutrophil lineage. MGG ×100.

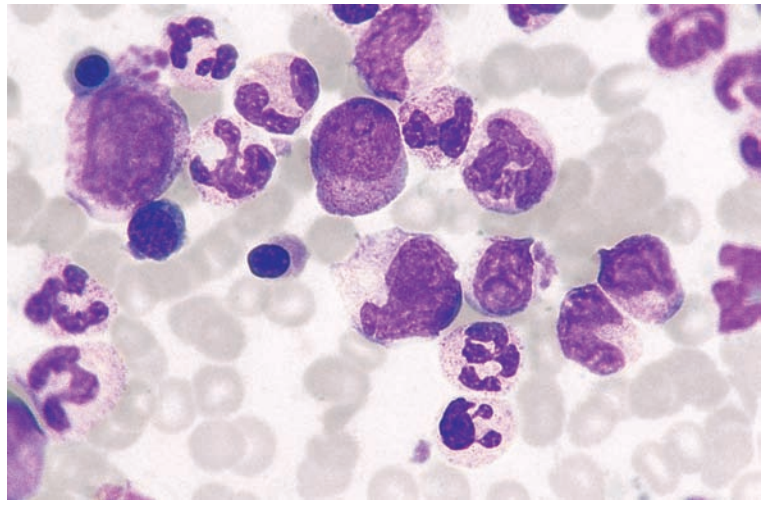


Fig. 5.29 BM film of a patient with aCML (same patient as Fig. 5.27) showing an increase of granulocytes and precursors with dysplastic features; there are also several cells of monocyte lineage. MGG ×100.

by definition, always less than 20% of circulating leucocytes. The NAP score is commonly decreased but in a minority of patients is increased.

The bone marrow findings reflect those in the peripheral blood. There is an increase of granulocytes and precursors but, in contrast to CGL, the M : E ratio is usually less than 10 : 1. Basophil and eosinophil precursors are less often increased whereas monocyte precursors are sometimes more prominent (Fig. 5.29). Megakaryocytes are reduced in about a third of patients and may be dysplastic (Fig. 5.30). Blasts may be somewhat increased but blasts plus promonocytes are less than 20%.

Atypical CML may terminate in blast crisis. This is usually a myeloid crisis but occasional lymphoid blast crises have been observed [111] suggesting an origin in a pluripotent stem cell.

Although aCML is usually readily distinguished from chronic phase CGL it can be difficult to make the distinction from CGL in early transformation

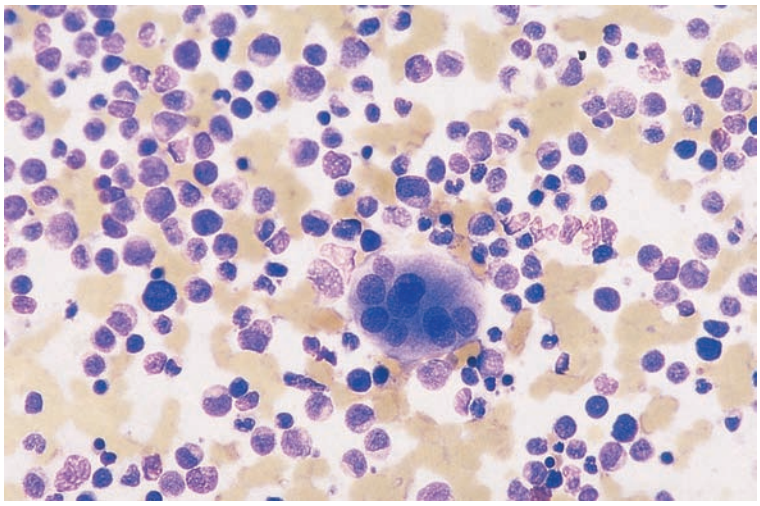


Fig. 5.30 BM film of a patient with aCML showing a multinucleated megakaryocyte. MGG ×40.

when there may be both dysplastic features and an atypical differential count. Cytogenetic and molecular genetic analysis may be necessary.

Making a distinction from CMML can be more difficult. Useful features are a higher WBC, a higher incidence of eosinophilia and basophilia, and the presence of larger numbers of circulating granulocyte precursors.

Cytogenetic and molecular genetic features

A number of clonal chromosomal abnormalities have been reported. No consistent association has been recognized although trisomy 8 and del(20q) are relatively common. There is no t(9;22)(q34;q11.2) or *BCR-ABL1* fusion and, by definition, no rearrangement of *PDGFRA*, *PDGFRB* or *FGFR1*. Mutations of *NRAS* and *KRAS* are common, one or other being present in about a third of patients. Inactivating mutations of *CBL* and *TET2* have been reported in 8% and 34%, respectively, of patients; these may be homozygous through recombination [112].

Problems and pitfalls

Because of the therapeutic implications, it is important not to misdiagnose CGL in accelerated phase as aCML. Cytogenetic and molecular analysis should therefore always be performed before making a diagnosis of aCML. Cytogenetic analysis is particularly important in any patient with aCML who has eosinophilia, in order not to miss patients who may have rearrangement of *PDGFRB*. Because of the considerably worse prognosis of aCML, it is also important to distinguish it from CMML.

Chronic myelomonocytic leukaemia

Chronic myelomonocytic leukaemia is an MDS/MPN with a monocyte count of greater than 1×10^9/l, no Ph chromosome or *BCR-ABL1* fusion gene, and fewer than 20% blasts plus promonocytes in the blood and marrow [113]. CMML is further divided into CMML-1, with blasts being less than 5% in the blood and less than 10% in the bone marrow, and CMML-2, with blasts being above these levels in blood, bone marrow or both, or with Auer rods being present. WHO diagnostic criteria are summarized in Table 5.7.

Clinical and haematological features

Patients are mainly elderly with a male predominance. The prevalence in the USA has been estimated at 0.37/100 000/year [114]. Patients usually present with symptoms of anaemia or with clinical features suggesting leukaemia (e.g. splenomegaly with or without hepatomegaly). A minority of patients have pleural, pericardial or peritoneal effusions, synovitis, lymphadenopathy or skin infiltration [115–117]. Rarely gum hypertrophy occurs [115]. Serum lysozyme is usually increased and urinary lysozyme is sometimes increased. Rare cases of renal failure may be related either to elevated urinary

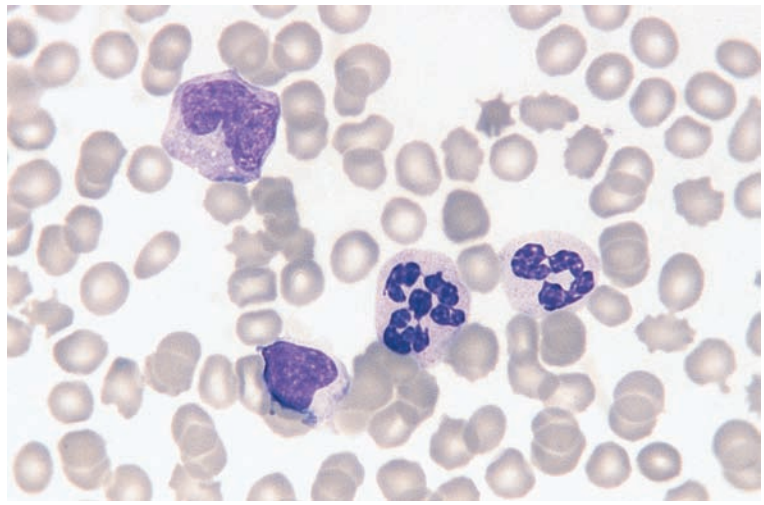


Fig. 5.31 PB film of a patient with chronic myelomonocytic leukaemia (CMML) showing a monocyte, a lymphocyte and two neutrophils, one of which is a macropolycyte. The red cells are poikilocytic and platelet numbers are reduced. MGG ×100.

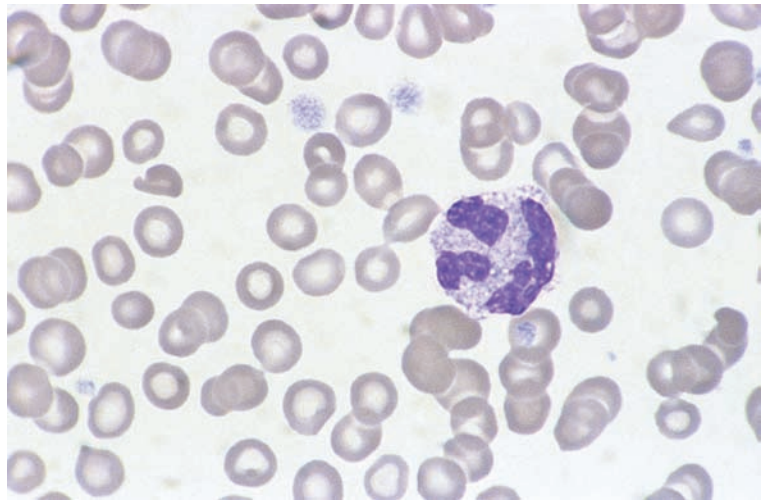


Fig. 5.32 PB film of a patient with CMML showing a binucleate macropolycyte. There is also anisocytosis, poikilocytosis, hypochromia and thrombocytopenia. MGG ×100.

lysozyme [117] or to renal infiltration. Associated immunological dysfunction is common. Immunoglobulin concentrations are increased in about a third of patients and 5–10% have a monoclonal protein; these patients can have an increased erythrocyte sedimentation rate. Autoantibodies are present in about a half of patients; these may include cold agglutinins. The direct antiglobulin test is positive in about 10% of patients.

The WBC ranges from normal to high. There is monocytosis (Figs 5.31 and 5.32) with the monocyte count being greater than 1×10^9/l. Monocytes are sometimes morphologically abnormal with hypersegmented or bizarre-shaped nuclei or with features of immaturity such as increased cytoplasmic basophilia and prominent cytoplasmic granules. Some promonocytes may be present but monoblasts are rarely seen. The neutrophil count is usually also elevated but this is not essential for the diagnosis and in some patients it is mildly reduced. Neutrophils sometimes show dysplastic features. There is sometimes eosinophilia. Anaemia may be present and is usually normocytic and normochromic. Macrocytosis may also occur and in patients with sideroblastic erythropoiesis, hypochromic microcytes and a dimorphic blood film are present.

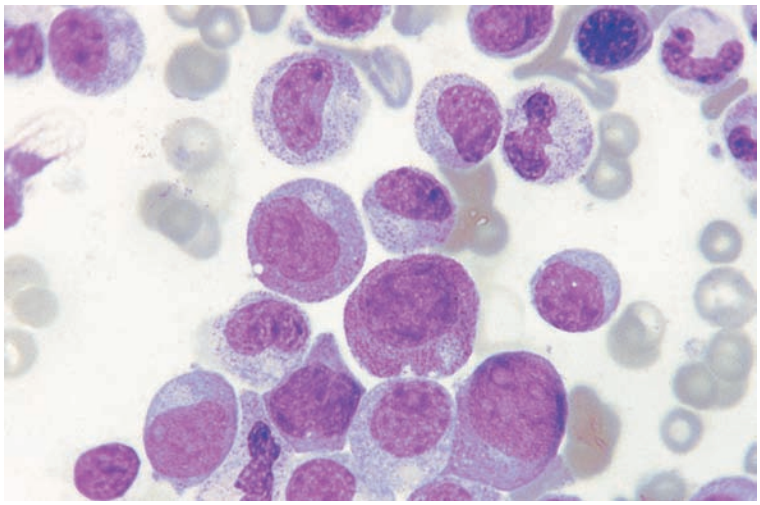


Fig. 5.33 BM film from a patient with CMML showing predominantly an increase in granulocytes and their precursors. MGG ×100.

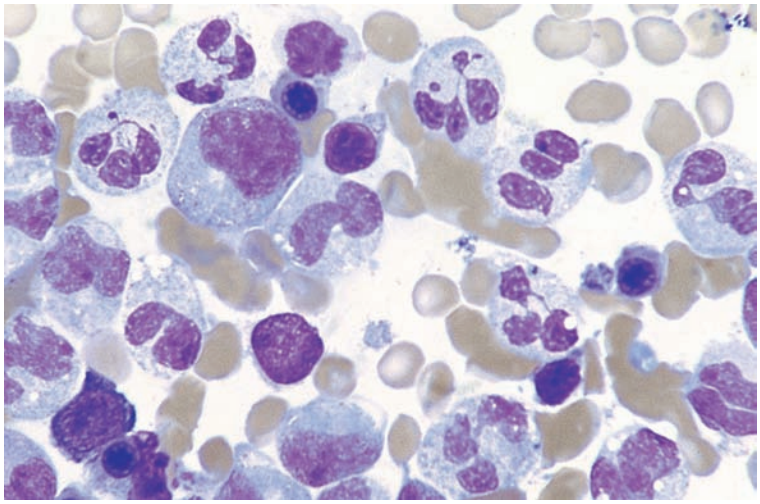


Fig. 5.34 BM film of a patient with CMML showing granulocytic dysplasia. The neutrophils are hypogranular and in addition there is a defect of nuclear lobulation with unusually long filaments separating lobes, an appearance showing some similarities to the inherited condition known as myelokathexis. MGG ×100. (By courtesy of Dr A. Copplestone, Plymouth.)

Patients with a positive direct antiglobulin test may have haemolysis with spherocytes in the blood film and those with increased immunoglobulins usually have increased rouleaux formation. The platelet count may be normal or low and dysplastic features may be present.

The bone marrow (Figs 5.33 and 5.34) is usually hypercellular. Immature monocytes may be prominent with there being relatively few mature monocytes. In some patients there is marked granulocytic hyperplasia with monocyte precursors being inconspicuous. Esterase cytochemistry may highlight the presence of larger numbers of cells of monocyte lineage than are appreciated on a Romanowsky stain. Individual cells may show reactivity for both CAE and non-specific esterase (NSE), activities that are normally characteristic of granulocytic and monocytic lineages, respectively. Blast cells vary from low levels to approaching 20%. Ring sideroblasts may be present and may exceed 15% of the erythroblasts. Dysplastic features may be observed in all lineages but, in some patients with CMML, dysplasia is minimal.

Trephine biopsy sections usually show a hypercellular marrow with increased granulocyte precursors. There may be foci of plasmacytoid dendritic

cells. Reticulin deposition is sometimes increased. Blasts are up to 20%.

The immunophenotype of peripheral blood monocytes often shows aberrant features such as reduced expression of CD14 and human leucocyte antigen DR (HLA-DR) and expression of CD56 [118]. Immunophenotyping can contribute to distinguishing CMML from reactive monocytosis, e.g. when aberrant expression of CD56 is present or when there are more than two aberrancies in antigen expressions [119].

The prognosis of CMML has been very variable between different series of patients (see Table 4.3 for the prognosis of patients whose disease was defined according to FAB criteria). The considerable variation in prognosis within the CMML group is likely to relate to how many patients with a high WBC (in some series indicative of worse prognosis) are included and to the wide range of bone marrow blast counts within this group (0% to approaching 20%). Other haematological features have also been found to be of prognostic significance. In one series of 213 FAB-defined cases, high WBC, monocyte count and lymphocyte count, low Hb, low platelet count, circulating granulocyte precursors and a higher bone marrow blast percentage were prognostically adverse [119]; in multivariate analysis, the independent prognostic features were an Hb below 120 g/l, circulating granulocyte precursors, a lymphocyte count of more than 2.5×10^9/l and bone marrow blast cells above 10% [120]. In a multivariate analysis of a second series of 212 patients, factors indicative of a worse prognosis were elevated lactate dehydrogenase, bone marrow blast cells above 10%, male sex, Hb below 120 g/l and a lymphocyte count of more than 2.5×10^9/l [121]. In a third series of patients, bone marrow eosinophilia and basophilia both correlated with worse prognosis with bone marrow basophilia being an independent risk factor for evolution to AML on multivariate analysis [122]. CMML-2 has a worse prognosis than CMML-1, again reflecting the prognostic significance of increased blast cells. An increase of CD34-positive cells in trephine biopsy sections is prognostically adverse.

Cytogenetic and molecular genetic features

A number of clonal cytogenetic abnormalities have been described including trisomy 8, monosomy 7, del(7q) and abnormalities of 12p. *RUNX1* mutations are common, in one series being found in 6 of 27 patients [123] and in another series in 30 of 81 patients [124]. *RAS* gene mutations are not infrequent, found in 4 of 35 patients in one series [125] and in 25 of 65 patients in another [120]. Mutations or deletion of *TET2*, a putative tumour suppressor gene, were found in 2 of 9 patients in one series [126] and in 50% in a larger series [112]. *CBL* inactivating mutations were found in 13% [112]. *JAK2* V617F mutation is uncommon, found in only 1 of 47 patients in one series [125].

Problems and pitfalls

It is important in the diagnosis of CMML that immature monocytes are not misclassified as promonocytes or misdiagnosis as acute monocytic leukaemia may occur. Promonocytes have a dispersed chromatin pattern. Cytogenetic analysis is essential in any patient with CMML who has eosinophilia, in order not to miss patients who may have rearrangement of *PDGFRB*.

Juvenile myelomonocytic leukaemia

This diagnostic group includes two childhood syndromes previously considered to have distinctive features: juvenile chronic myeloid leukaemia and the infantile monosomy 7 syndrome. They are now considered part of the spectrum of a single disorder, characterized by both dysplastic and proliferative features and by mutation of one of a group of genes involved in a single signalling pathway. Causative mutations may occur in a pluripotent lymphoid–myeloid haemopoietic stem cell [127]. The WHO criteria for this diagnosis are summarized in Table 5.8 [128]. Predisposing conditions include neurofibromatosis type 1 (*NF1* mutation) and Noonan syndrome (*PTPN11* mutation). However, it should be noted that a significant proportion of children with Noonan syndrome have abnormal haemopoiesis resembling juvenile myelomonocytic leukaemia (JMML) that is transient [129]; they can also have isolated monocytosis. The JMML/transient myeloproliferative disorder of Noonan syndrome is associated mainly with sporadic rather than familial cases and with a different group of mutations [130,131]. As in the case of Down

Table 5.8 WHO criteria for a diagnosis of juvenile myelomonocytic leukaemia [128].

- Monocyte count greater than 1×10^9/l
- Blasts plus promonocytes less than 20% in peripheral blood and bone marrow
- No Ph chromosome or *BCR-ABL1* fusion gene
- Two or more of the following
  - Haemoglobin F percentage increased for age
  - Immature granulocytes in the peripheral blood
  - White cell count greater than 10×10^9/l
  - Clonal chromosomal abnormality present
  - Myeloid progenitors hypersensitive to GM-CSF *in vitro*

Ph, Philadelphia (chromosome); GM-CSF, granulocyte–monocyte colony-stimulating factor.

syndrome, the transient nature of the abnormality does not exclude the possibility that it is neoplastic in nature. JMML is rare, the incidence being about 1.8 cases/1 000 000/year [131].

Clinical and haematological features

Onset is usually in infancy (median age 2 years) and there is a male predominance. Common clinical features include fever, anaemia, hepatomegaly, marked splenomegaly, lymphadenopathy, an eczematous or maculopapular rash, xanthomas and a bleeding tendency. Infections, including tonsillitis and bronchitis, are common. Some children will have the physical features of Noonan syndrome (facial dysmorphism and cardiac anomalies) and others will have *café-au-lait* spots, as a result of type 1 neurofibromatosis.

The peripheral blood (Fig. 5.35) shows leucocytosis, neutrophilia and prominent monocytosis. Granulocyte precursors including blasts are often present. Some cases have eosinophilia or basophilia. Anaemia, thrombocytopenia and circulating nucleated red blood cells are common. Macrocytosis may be present, particularly in those with monosomy 7. Microcytosis is sometimes present but in the majority of cases, red cells are normocytic.

The bone marrow is hypercellular with granulocytic hyperplasia and usually an increase in monocytes and their precursors, eosinophils or basophils. Recognition of the monocytic component may require cytochemical stains. There may be erythroid hyperplasia. Megakaryocytes are often reduced in number. The blast percentage is often somewhat elevated. Trilineage dysplasia may be present.

There may be reversion to some characteristics of fetal erythropoiesis (Fig. 5.36). The haemoglobin F level, glucose-6-phosphate dehydrogenase activity and the expression of i antigen are increased while the haemoglobin A_2 percentage, carbonic anhydrase activity and the expression of I antigen are reduced. The $^{G}\gamma^{A}\gamma$ ratio of haemoglobin F is similar to that in the neonatal period and when the

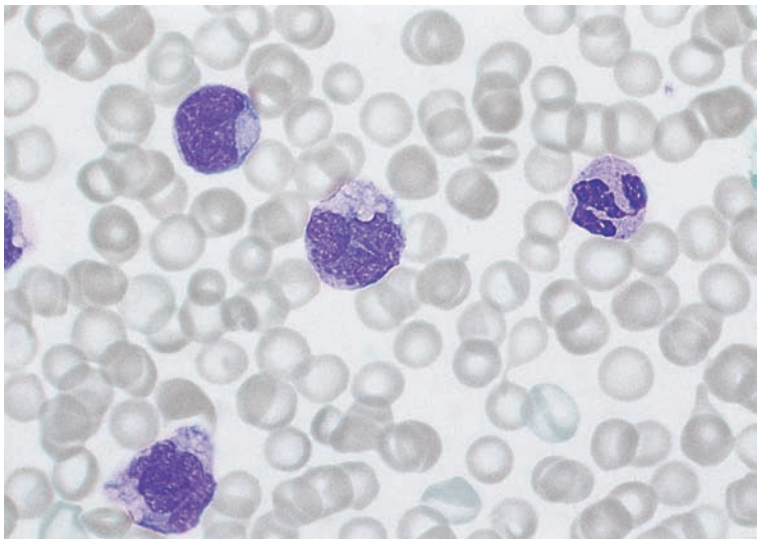


Fig. 5.35 PB film of a child with juvenile myelomonocytic leukaemia (JMML) showing a blast cell and several cytologically abnormal monocytes. MGG ×100.

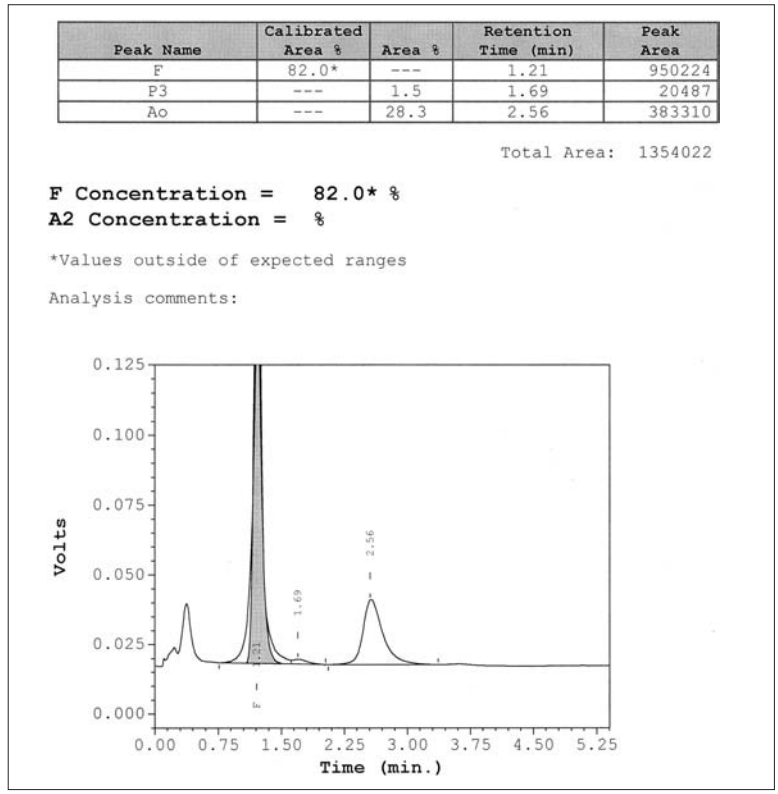


Fig. 5.36 High performance liquid chromatography on a Bio-Rad Variant II instrument in a child with JMML showing an increase of haemoglobin F to 70–80% of total haemoglobin and a total absence of haemoglobin A_2, both these abnormalities being characteristic of fetal erythropoiesis.

haemoglobin F percentage is significantly increased, the oxygen dissociation curve is left shifted. It should be noted that haemoglobin F is elevated at birth and in normal infants may take 6–12 months to fall to adult levels. This should be considered when infants with an apparent MDS/MPN present in early infancy. The concentration of serum immunoglobulin is increased in 50–80% of patients and some have a positive direct antiglobulin test or other autoantibodies. Serum lysozyme is increased.

Spontaneous colony growth from peripheral blood or bone marrow cells [132], resulting from hypersensitivity to granulocyte–macrophage colony-stimulating factor (GM-CSF), is characteristically present and is an important diagnostic criterion.

Although blast transformation occurs in only a minority of cases [133] the prognosis is poor, particularly in those with an onset after the age of 6–12 months. Unless bone marrow transplantation is carried out the median survival is less than a year [134]. Bad prognostic features include a later age of onset (e.g. more than 2 years of age), a low platelet count (e.g. 33×10^9/l or less) and an elevated percentage of haemoglobin F (e.g. 15% or higher) [128,134].

Cytogenetic and molecular genetic features

Most cases are cytogenetically normal at diagnosis, although a variety of clonal chromosomal abnormalities have been described, either at diagnosis or during disease progression. Either trisomy 8 or a complex karyotypic abnormality occurs in about 10% of patients and as many as a quarter of patients have monosomy 7 at presentation or develop it during the course of the disease.

Molecular genetic abnormalities observed include a high frequency of *NRAS* and *HRAS* mutations, present in about a quarter of children [131,135]. Children with neurofibromatosis constitute around 11% of cases of JMML [131]; they often show loss of the normal *NF1* allele, which may be the result of acquired uniparental disomy [136,137]. Other children may also have biallelic inactivation of this gene. Children with Noonan syndrome are a minority of

cases of JMML but as many as a third of other children with JMML have a somatic mutation of *PTPN11*. The specific mutations in these cases tend to differ from those in JMML associated with Noonan syndrome [130]. Neurofibromin, the protein encoded by *NF1*, is important in regulating the *RAS* family of oncogenes and *PTPN11* encodes SHP-2, a protein tyrosine phosphatase that functions in GM-CSF receptor–RAS signalling [131]. The three most common molecular genetic abnormalities are thus closely related. They are mutually exclusive and one or other occurs in three quarters of cases.

Problems and pitfalls

It is necessary to be aware that viral infection in infants (cytomegalovirus, Epstein–Barr virus, human herpesvirus 6, human herpesvirus 8, parvovirus B19) can simulate JMML, even to the extent of leading to spontaneous growth of granulocyte–macrophage colonies [127]. In infants with Noonan syndrome it should be noted that a syndrome resembling JMML may be transient.

Conclusions

The chronic myeloid leukaemias include conditions that are primarily proliferative (MPN in the WHO classification) and other conditions that also have dysplastic features (MDS/MPN in the WHO classification). With advances in knowledge, classification of this group of disorders is increasingly being based on the underlying cytogenetic and molecular genetic abnormality. Understanding the molecular basis of the leukaemia is important not only for our understanding of the nature of these conditions but also for indicating possible therapeutic choices. There is already specific targeted therapy for leukaemias associated with *BCR-ABL1*, *FIP1L1-PDGFRA* and other fusion genes involving *PDGFRA* or *PDGFRB*. Further therapeutic advances are likely to follow a better molecular understanding of other chronic myeloid leukaemias.

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SIX

LYMPHOID LEUKAEMIAS OF MATURE T, B AND NATURAL KILLER CELLS

Diagnosis and classification of lymphoid leukaemias and leukaemic-phase lymphomas of mature T, B and NK cells

The term leukaemia ('white blood') refers to a haemopoietic or lymphoid neoplasm that involves the bone marrow, and usually also the blood, whereas a lymphoma is a lymphoid neoplasm that presents as a tumour of extramedullary tissues. The distinction is, to some extent, artificial. For example, Burkitt lymphoma can present either as a jaw tumour or as a leukaemia with blood and marrow involvement. Adult T-cell leukaemia/lymphoma presents as leukaemia in about 90% of patients but as lymphoma in 10%. Chronic lymphocytic leukaemia has a lymphomatous equivalent, known as small lymphocytic lymphoma. This chapter deals both with conditions that are usually regarded as chronic lymphoid leukaemias and with others which, although they are usually regarded as lymphomas, can present as leukaemia or develop a leukaemic phase during the course of the illness [1]. Although this group of disorders are often referred to as chronic lymphoid leukaemias it should be noted that this is not necessarily a satisfactory description of their clinical behaviour. They are more accurately regarded as neoplasms of mature B, T or NK (natural killer) cells. In some instances the clinical course is as rapidly progressive as that of the precursor T and B neoplasms that constitute acute lymphoblastic leukaemia/lymphoblastic lymphoma.

The likelihood of leukaemic manifestations varies between different types of non-Hodgkin lymphoma (NHL). For example, peripheral blood involvement

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is frequent in mantle cell lymphoma and to a lesser extent in follicular lymphoma but is quite uncommon in diffuse large B-cell lymphoma. The clinical features of lymphoma in leukaemic phase are determined largely by the nature of the underlying lymphoma. They usually include lymphadenopathy, splenomegaly or both, although occasional cases are diagnosed incidentally from a blood film before any organomegaly has occurred.

The classification for lymphoid leukaemias and leukaemic phase lymphomas of mature lymphocytes is ideally based on cytological, immunophenotypic, histological and cytogenetic/molecular genetic features, although not all techniques need to be employed in all patients. In the 2008 World Health Organization (WHO) classification these techniques provide the basis for precise categorization [2].

Cytological and immunophenotypic features are of major importance in the diagnosis and further categorization of lymphoid leukaemias. Neither provides a reliable diagnosis without the other. Sometimes precise diagnosis also requires trephine biopsy histology, lymph node or splenic histology or cytogenetic or molecular genetic analysis. Cytochemistry also has a role, albeit minor.

Cytology

Assessment of cytological features is usually best carried out on peripheral blood films but can also be done on films prepared from aspirates of bone marrow or other tissues or on imprints from trephine biopsy or other tissue biopsy specimens.

Immunophenotyping

Immunophenotyping is essential for establishing if a leukaemia is of B, T or NK lineage and, when there is any diagnostic difficulty, will also help to distinguish acute lymphoblastic leukaemia (ALL) and lymphoblastic lymphoma from lymphoid leukaemias of mature cells. In addition, many chronic lymphoproliferative disorders have an immunophenotype that is sufficiently characteristic to be useful in making a specific diagnosis. Immunophenotyping can also provide prognostic information and can provide a basis for monitoring minimal residual disease (MRD).

Providing evidence of clonality is an important function of immunophenotyping since clonality provides presumptive evidence of a neoplastic rather than a reactive condition. In the case of B-lineage leukaemias, light chain restriction, i.e. expression of either κ or λ light chain but not both, usually indicates a monoclonal population. Although monoclonal B cells show light chain restriction, they may express more than one type of heavy chain (μ, δ, α, γ). It should also be noted that expansion of clones of CD5-positive (CLL-like) and CD5-negative B cells is quite common in the elderly [3]; it cannot be inferred from the presence of a small clone that there is a clinically significant disorder.

Specific panels of monoclonal antibodies (McAb) have been recommended for the initial assessment and for the further characterization of chronic lymphoproliferative disorders [4–6] (Tables 6.1 and 6.2). Immunophenotyping is now generally carried out by flow cytometry, supplemented by immunohistochemistry on biopsy sections.

The role of immunophenotyping in the investigation of lymphoproliferative disorders can be summarized as follows:

1 Assessment of lineage.

2 Presumptive demonstration of clonality.

3 Recognition of immunophenotypic characteristics that distinguish the chronic lymphoproliferative disorders from ALL.

4 Demonstration of patterns of antigen expression that support a specific diagnosis.

5 Recognition of different patterns of antigen expression within a specific disease category that are of prognostic significance, e.g. overexpression of the tumour suppressor gene, *TP53*, which often indicates gene mutation, or expression of CD38 or zeta-associated protein 70 (ZAP70), indicative of a worse prognosis in chronic lymphocytic leukaemia (CLL).

6 Confirmation of expression of a specific antigen (e.g. CD20 or CD52) when a McAb is to be used in therapy.

7 Monitoring of MRD, e.g. in CLL monitoring of CD19-positive, CD5-positive, CD20-weak and CD79b-weak cells.

Most antigens detected in flow cytometry are expressed on the surface membrane but expression of cyclin D1 and p53 is nuclear, so cells must be 'permeabilized' if these antigens are to be detected. It is similarly necessary to permeabilize cells if a McAb is to detect a cytoplasmic antigen (e.g. κ or λ light chain) or a cytoplasmic epitope of a transmembrane antigen (e.g. CD79a).

Gating of a particular population of cells can be useful when it is probable that any neoplastic clone

Table 6.1 Panel of monoclonal antibodies recommended by the US–Canadian Consensus group for immunophenotyping in chronic lymphoproliferative disorders [5].

Lineage	Core panel	Supplementary panel
B	CD5, CD10, CD19, CD20, κ, λ	CD11c, CD22, CD23, FMC7
T/NK cell	CD3, CD4, CD5, CD7, CD8	CD2, CD16, CD56, CD57, TCRαβ, TCRγδ
Non-lineage restricted	CD45	CD25, CD38, BB4 (CD138)

NK, natural killer; TCR, T-cell receptor.

Table 6.2 Panel of monoclonal antibodies recommended by the British Committee for Standards in Haematology (BCSH) for immunophenotyping in chronic lymphoproliferative disorders [6].

Lineage	Core panel	Supplementary panel
B	CD19, CD23, CD22, CD79b, FMC7, surface membrane κ and λ	CD11c, CD103, HC2*, cytoplasmic κ and λ, CD79a, CD138, cyclin D1
T/NK cell	CD2, CD5	CD3, CD7, CD4, CD8, CD11b, CD16, CD56, CD57, TIA-1
Non-lineage restricted	CD5	CD25, TdT

NK, natural killer; TdT, terminal deoxynucleotidyl transferase.
* Not commercially available so CD123 can be substituted.

is likely to be only a minor proportion of the cells present. For example, gating on CD138-positive cells can be useful in studying plasma cells. Similarly, if there appear to be two populations of B cells present they should be analysed individually; CD22 expression can be useful for this since neoplastic B cells may under- or overexpress CD22 permitting analysis of κ : λ ratios separately on the normal and the abnormal population [7]. Gating may be essential if it is necessary to study expression of an antigen on neoplastic cells that is also expressed on normal cells, e.g. in studying ZAP70 expression on CLL B cells.

Histology

In selected cases, histology of bone marrow, lymph node, spleen or skin can be useful in diagnosing chronic lymphoid leukaemias and in distinguishing them from NHL. Characteristic patterns of infiltration observed in trephine biopsy sections and the terms conventionally used to describe them are shown in Fig. 6.1 [8]. It should be noted that the term 'interstitial' indicates that leukaemic cells are infiltrating between the normal haemopoietic cells without disturbing the structure of the bone marrow. Conventionally the term 'diffuse' is used only to designate heavy infiltration that obliterates the normal bone marrow architecture; the term 'packed marrow' has also been used to describe this pattern of infiltration. Histology can be supplemented by immunohistochemistry. This is essential in patients with only small numbers of neoplastic cells in the peripheral blood or bone marrow aspirate in whom immunophenotyping of cells in suspension is therefore not possible.

Cytogenetic and molecular genetic analysis

Cytogenetic analysis is sometimes useful in establishing clonality and in confirming the neoplastic nature of a lymphoproliferative disorder. More often it is useful in indicating a precise diagnosis since there are certain recurrent cytogenetic abnormalities that are characteristic of particular leukaemias or lymphomas. Karyotypic abnormalities may be detected by conventional cytogenetic analysis or by fluorescence *in situ* hybridization (FISH) or other *in situ* hybridization techniques.

Molecular genetic analysis is useful in establishing clonality by the detection of immunoglobulin (*IGH*) or T-cell receptor (*TCR*) locus rearrangement. It is also useful in making a specific diagnosis since it is a means of identifying characteristic molecular rearrangements present in different subtypes of leukaemia or lymphoma. Molecular genetic analysis can be used for the monitoring of MRD. The most useful techniques are polymerase chain reaction (PCR), for analysis of genomic deoxyribonucleic acid (DNA), and reverse transcriptase PCR (RT-PCR), for detection of specific messenger ribonucleic acid (RNA) transcripts. Immunocytochemistry and immunohistochemistry can be regarded as extensions of molecular genetic techniques when they

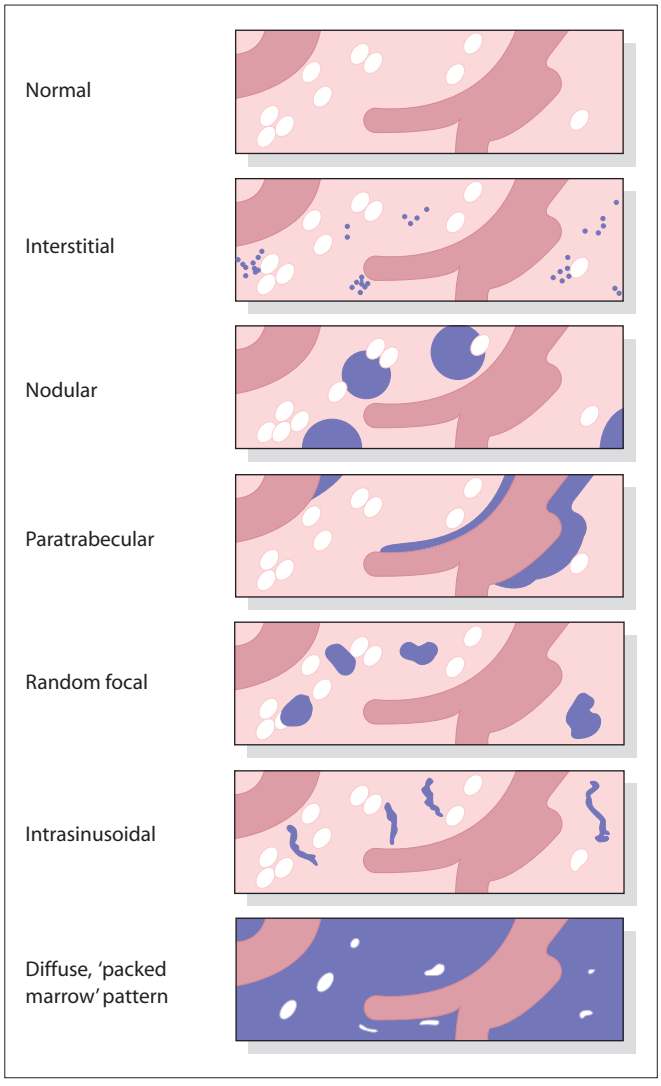


Fig. 6.1 Patterns of bone marrow (BM) infiltration observed in lymphoproliferative disorders (reproduced with permission from 8).

are used to identify the product of a specific gene. The cytogenetic and molecular genetic abnormalities most characteristic of various chronic lymphoid leukaemias are summarized in Table 6.3.

The latest molecular genetics tool to be applied to diagnosis and classification of lymphoid leukaemias and lymphomas is gene expression analysis by microarray analysis. Already, characteristic patterns can be recognized for CLL/small lymphocytic lymphoma, mantle cell lymphoma, marginal zone B-cell lymphoma and three subtypes of diffuse large B-cell lymphoma [9,10].

Staging and prognostic scoring indices

Non-Hodgkin lymphoma in adults is staged by the Ann Arbor staging system, initially proposed for Hodgkin lymphoma (Table 6.4; Fig. 6.2). By definition, lymphomas that involve the peripheral blood are stage IV. A different staging system if applied to CLL (see below) and also to Burkitt lymphoma and cutaneous lymphomas.

Various prognostic scoring systems have been proposed, e.g. for aggressive non-Hodgkin lymphoma [11] (Table 6.5) and for follicular lymphoma [12] (Table 6.6).

Table 6.3 Cytogenetic and molecular genetic abnormalities most characteristic of chronic lymphoid leukaemias and lymphomas with a leukaemic phase.

Cytogenetic abnormality	Associated molecular genetic abnormality	Approximate frequency (where known)
Chronic lymphocytic leukaemia		
Deletion or rearrangements at 13q12 or 13q14	Sometimes deletion of *RB1* (13q14.1-q14.2), *DBM* (13q14) or *BRCA2* (13q12.3)	50%
del(11)(q22.3)	Deletion of *ATM*	20%
Trisomy 12	Unknown	20%
del(17)(p13.1)	Deletion of *TP53*	10%
del(6)(q21)	Unknown	5%
Prolymphocytic leukaemia		
Often complex, may include trisomy 3, trisomy 12, del(6q), del(7q), monosomy 7, del(11)(q23), del(13q14.3)	As above or unknown	
Splenic marginal zone lymphoma (splenic lymphoma with villous lymphocytes)		
Trisomy 3	Unknown	20%
Follicular lymphoma		
t(14;18)(q32;q21)	Dysregulation of *BCL2* by proximity to *IGH* locus	70–90%
t(2;18)(p12;q21)	Dysregulation of *BCL2* by proximity to κ locus	
t(18;22)(q21;q11.2)	Dysregulation of *BCL2* by proximity to λ locus	
Mantle cell lymphoma		
t(11;14)(q13;q32)	Dysregulation of *BCL1* by proximity to *IGH* locus, with consequent overexpression of cyclin D1 in the nucleus	90%
Lymphoplasmacytoid lymphoma		
t(9;14)(p13;q32)	Dysregulation of *PAX5* by proximity to *IGH* locus	Minority and not specific
Burkitt lymphoma		
t(8;14)(q24;q32)	Dysregulation of *MYC* by proximity to *IGH* locus	70–80%
t(2;8)(p12;q24)	Dysregulation of *MYC* by proximity to κ locus	
t(8;22)(q24;q11.2)	Dysregulation of *MYC* by proximity to λ locus	
T-cell prolymphocytic leukaemia		
inv(14)(q11q32), t(14;14)(q11;q32)	*TCL1A* and *TCL1B* dysregulated by proximity to *TCRAD* locus	75%
t(X;14)(q28;q11)	*MTCP1* dysregulated by proximity to *TCRAD* locus	
t(X;7)(q28;q35)	*MTCP1* dysregulated by proximity to *TCRB* locus	

Chronic leukaemias/lymphomas of B lineage

Chronic leukaemias of B-lymphocyte lineage express monoclonal immunoglobulin on the cell surface membrane or, less often, in the cytoplasm. This may be a complete immunoglobulin or there may be expression of only heavy chain or only light chain. Neoplastic B cells also express a variety of antigenic markers, some of which are shared with T lymphocytes or with haemopoietic cells, and others of which are more specific, within the haemopoietic and lymphoid lineages, for B lymphocytes (Table 6.7). Some of the immunophenotypic markers of leukaemic and normal lymphocytes are pan-B (characteristically positive with all B-lineage lymphocytes), some are pan-mature B, and some show selectivity for subsets of normal B lympho-

Table 6.4 Staging of Hodgkin and non-Hodgkin lymphoma.

Stage	Criteria
I	Disease in one lymph node region or lymphoid structure (e.g. thymus, spleen or Waldeyer's ring); stage I_E has limited contiguous extension beyond a lymph node but with this being encompassable in a radiotherapy field
II	Disease in two or more lymph node regions or structures but confined to one side of the diaphragm
III	Disease on both sides of the diaphragm but confined to lymph nodes and lymphoid structures
IV	Spread (other than limited contiguous extension) beyond lymph nodes and spleen, e.g. to liver, lung or bone marrow
A	Having no B symptoms
B	Having (i) loss of more than 10% of body weight in the preceding 6 months, (ii) drenching night sweats, (iii) fever >38°C

Each patient is given a composite stage, e.g. IA, IIIB.

Table 6.5 International Prognostic Index for high-grade non-Hodgkin lymphoma [11].

Criterion	Score
Age >60 years	1
Stage III or IV	1
More than one extranodal site	1
Bedridden for some or all of the day (cf. ambulatory with or without symptoms)	1
Lactate dehydrogenase above normal	1

Scores are added: 0–1, low risk; 2, low intermediate risk; 3, high intermediate risk; 4–5, high risk.

Table 6.6 Follicular Lymphoma International Prognostic Index (FLIPI) [12].

Criterion	Score
Age >60 years	1
Stage III or IV	1
Haemoglobin concentration <120 g/l	1
More than four nodal areas involved	1
Lactate dehydrogenase above normal	1

Scores are added: 0–1, low risk; 2 intermediate risk; 3–5 high risk.

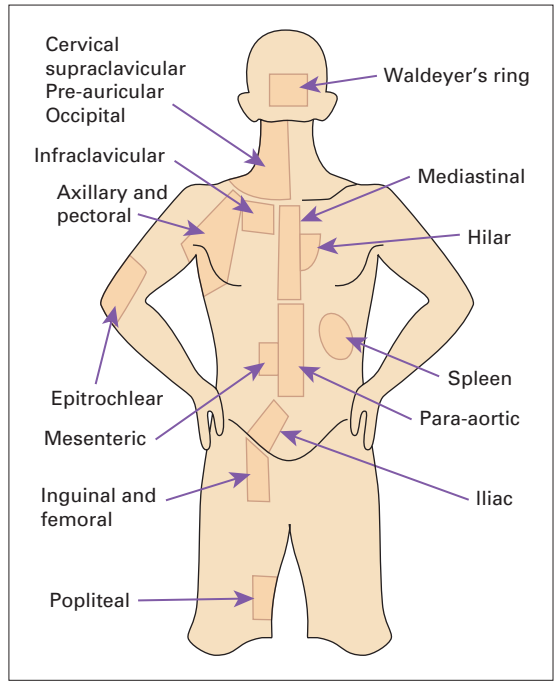


Fig. 6.2 Lymph node regions used for staging of Hodgkin and non-Hodgkin lymphoma.

cytes and for cells in specific lymphoproliferative disorders. Chronic B-lineage lymphoid leukaemias do not express terminal deoxynucleotidyl transferase (TdT) or CD34, whereas lymphoblasts generally express TdT and sometimes express CD34. Conversely, lymphoma cells usually express surface membrane immunoglobulin (SmIg) and an antigen recognized by the FMC7 McAb, whereas lymphoblasts do not.

DNA analysis shows that, in B-lineage chronic lymphoid leukaemias, the heavy chain and usually the light chain immunoglobulin loci have undergone rearrangement; in some cases the T-cell receptor β (*TCRB*) locus has also been rearranged.

The chronic leukaemias of B lineage can be further categorized as discussed below. Characteristic immunophenotypic markers of each disease entity are shown in Table 6.8.

Chronic lymphocytic leukaemia

Chronic lymphocytic leukaemia is a chronic B-lineage lymphoproliferative disorder defined by characteristic morphology and immunophenotype. The cell of origin is now thought to be an

Table 6.7 Some monoclonal antibodies used in the characterization of chronic lymphoid leukaemias of B lineage.

Cluster designation or other specificity	Specificity within haemopoietic and lymphoid lineages
CD19, CD20, CD24	B lineage; CD19 and CD24 are expressed early in B-lineage differentiation, CD20 later; CD24 is also expressed by neutrophils
CD21	Complement receptor CR2 (C3dR) and also receptor for Epstein–Barr virus: subset of normal B cells, cells of majority of cases of CLL and about 50% of cases of B-NHL; also expressed by follicular dendritic cells
CD22	Most mature B cells and some B-cell precursors; cells of NHL, HCL and B-PLL, weakly expressed in CLL
CD23	Low-affinity FcεR: expressed on activated B cells, cells of the majority of cases of CLL and CLL/PL and a minority of cases of B-PLL and B-NHL; also expressed on eosinophils, follicular dendritic cells and platelets
CD79a	Part of an immunoglobulin-associated heterodimeric membrane protein; expressed in cells of most B-cell lymphoproliferative disorders, both mature and immature
CD79b	Part of an immunoglobulin-associated heterodimeric membrane protein; expressed on normal B cells and in the majority of cases of most B-lineage lymphoproliferative disorders; however, expressed in only a half of lymphoplasmacytoid lymphoma, a quarter of cases of HCL and only a small minority of cases of CLL
CD5*	Expressed on thymocytes and T lymphocytes and in many T-cell malignancies; expressed on a small subset of normal B cells, in a majority of cases of B-CLL and mantle cell lymphoma and in a minority of cases of B-PLL
CD10	Common ALL antigen but also expressed on some B-NHL, particularly follicular lymphomas and some plasma cell leukaemias and myelomas; more weakly expressed by some T-lineage ALL; expressed on some bone marrow stromal cells
CD11c	Hairy cells, B-PLL and some cases of hairy cell leukaemia variant, also some B, T, NK and myeloid cells
CD25*	Interleukin 2 receptor: expressed on activated T and B cells, monocytes, hairy cells and ATLL cells
CD43*	Expressed on T cells and activated B cells; expressed in CLL/small lymphocytic lymphoma and mantle cell lymphoma but not in follicular lymphoma, prolymphocytic leukaemia or hairy cell leukaemia; sometimes expressed in Burkitt lymphoma, lymphoplasmacytic lymphoma and marginal zone lymphoma
CD38*	Early or activated T and B cells, subset of CLL, haemopoietic precursors, thymic cells, plasma cells including myeloma cells
CD103	Intra-epithelial lymphocytes, small subset of peripheral blood lymphocytes, hairy cells
CD123	Hairy cells, blastic plasmacytoid dendritic cell neoplasm
CD138	Plasma cells including myeloma cells
FMC7	Subset of normal mature B cells (30–60%), cells of majority of cases of B-NHL, HCL and B-PLL but not CLL or B-lineage ALL (unclustered but appears to recognize a conformational epitope of CD20 [13])
HLA-DR*	Virtually all B lymphocytes and their precursors, activated T cells, haemopoietic precursors, monocytes (unclustered)
ZAP70*	Subset of CLL cases, also T cells, NK cells, a small minority of normal B cells, mast cells and basophils
Anti-Ig, anti-γ, anti-α,† anti-μ, anti-δ, anti-κ, anti-λ†	Immunoglobulin and its constituent chains: SmIg is a pan-mature B-cell marker; cytoplasmic heavy chain of IgM is detectable in pre-B cells (cμ) and in plasma cells (cIg); anti-γ, α, μ and δ identify subsets of B cells and anti-κ and anti-λ are useful for demonstrating clonality

ALL, acute lymphoblastic leukaemia; ATLL, adult T-cell leukaemia lymphoma; B-CLL, B chronic lymphocytic leukaemia B-NHL, B non-Hodgkin lymphoma; B-PLL, B-prolymphocytic leukaemia; CLL, chronic lymphocytic leukaemia; CLL/PL, CLL, mixed cell type; HCL, hairy cell leukaemia; NHL, non-Hodgkin lymphoma; NK, natural killer; SmIg, surface membrane immunoglobulin; ZAP70, zeta-associated protein 70.

* Also positive in some or most T lymphocytes.

† Some polyclonal antisera are in current use.

Table 6.8 Characteristic immunophenotype of chronic B-cell leukaemias and B-cell lymphomas that can involve the peripheral blood.

Marker	CLL	PLL	HCL	Follicular lymphoma	Mantle cell lymphoma	SMZL/ SLVL	Plasma cell leukaemia
SmIg	Weak	Strong	Strong or moderate	Strong	Moderate	Strong	Negative
CyIg	–	–/+	–/+	–	–	–/+	++
CD5	++	–/+	–	–	++	–	–
CD19, 20, 24, 79a	++*	++	++†	++	++	++	–
CD79b	–	++	–/+	++	++	++	–
CD23	++	–	–	–/+	–/+	–/+	–
FMC7, CD22	–/+	++	++	++	+	++	–
CD10	–	–/+	–	+	–/+	–	–/+
CD11c	–/+	++	++	–	–/+	+	?
CD25	–/+	–	++	–	–	–/+	–
CD38	–/+	–	–/+	–/+	–	–/+	++
HLA-DR	++	++	++	++	++	++	–

The frequency with which a marker is positive in >30% of cells in a particular leukaemia is indicated as follows: ++, 80–100%; +, 40–80%; –/+, 10–40%; –, 0–9%.
CLL, chronic lymphocytic leukaemia; CyIg, cytoplasmic immunoglobulin; HCL, hairy cell leukaemia; PLL, prolymphocytic leukaemia; SLVL, splenic lymphoma with villous lymphocytes; SmIg, surface membrane immunoglobulin; SMZL, splenic marginal zone lymphoma.
* CLL cells express CD20 fairly weakly.
† HCL cells are negative with at least some monoclonal antibodies of the CD24 cluster.

antigen-experienced, activated B cell, which in some patients is a post-germinal centre B cell that has undergone somatic hypermutation of *IGVH* genes and in others is a cell that has responded to antigen by a T-cell-independent process outside the germinal centre and without hypermutation. The normal equivalent of the CLL cell may thus be a memory B cell. Small lymphocytic lymphoma is an equivalent lymphoma without there being circulating neoplastic cells in large enough numbers to satisfy the criteria for CLL [14]. In most patients CLL is preceded by monoclonal B-cell lymphocytosis (see page 320). In a retrospective study of 45 patients for whom cryopreserved lymphocytes were available, a monoclonal population was detected by flow cytometry in 42 (93%) and when molecular analysis was added a clone was detected in 44 patients (98%); clones were detected a median of 3 years (range 3–77 months) before the diagnosis of CLL was made [15]. However, prolonged lymphocytopenia, possibly representing reduced numbers of T lymphocytes, has also been reported preceding CLL [15a]. Mu (μ) heavy chain disease resembles CLL but is classified separately.

Clinical, haematological and cytological features

Chronic lymphocytic leukaemia is the commonest leukaemia in Western Europe and North America with an incidence in different surveys varying between 1 and more than 10/100 000/year. On average the incidence in European countries and North America is of the order of 3.5/100 000/year. The incidence is lower in Chinese, Japanese, Indians and North American Indians and is higher in Jews. The incidence in those of African ancestry is intermediate between the rates in Asian and white populations [16]. The prognosis is worse in South Asians (Indians) and onset may be at a younger age [17]. CLL is typically a disease of the elderly with a higher incidence in males. The ratio of men to women is about 2–3 : 1. Some cases are familial, the pattern of inheritance suggesting a dominantly acting gene [18]. In some families there is linkage to 13q21.33-q22.2 [19]. In the later stages, CLL is characterized by lymphadenopathy, hepatomegaly, splenomegaly, recurrent infection and eventually impairment of bone marrow function. In the early stages of the disease there are no symptoms or abnormal physical findings and the

diagnosis is made incidentally. Incidental diagnosis now occurs in 70–80% of patients. Various arbitrary levels of absolute lymphocyte count have been suggested for the diagnosis of CLL (for example, greater than 10×10^9/l) but the demonstration of a monoclonal population of B lymphocytes with a characteristic immunophenotype permits diagnosis at an earlier stage when the lymphocyte count is less elevated. A 2007 International Workshop on Chronic Lymphocytic Leukemia, which updated the 1996 National Cancer Institute guidelines, suggested that a lymphocyte count of at least 5×10^9/l was sufficient for diagnosis when the immunophenotype was typical and clonality was demonstrated [20]. The 2008 WHO classification has similar criteria, requiring a minimum of at least 5×10^9/l clonal B cells [14]. Some patients have a paraprotein present in low concentration. Spontaneous remission can occur in CLL but this is a rare occurrence [21]; when it occurs, the leukaemic clone may reduce to minimal levels [21].

The leukaemic cells in CLL are typically small with a conspicuous though usually narrow rim of cytoplasm (Fig. 6.3). Cells are more uniform in their characteristics than are normal peripheral blood lymphocytes. The nuclear and cytoplasmic outlines are generally regular, although some cases have somewhat indented nuclei. Nuclear chromatin is dense and clumped with nucleoli visualized poorly if at all on light microscopy. The clumping of chromatin often gives the nucleus a mosaic pattern. Cytoplasm is weakly basophilic and sometimes contains small vacuoles and, occasionally, crystals or granules [22]. CLL lymphocytes are more fragile than normal lymphocytes and thus the formation of smudge cells or smear cells during the spreading of the blood film is common; this feature can be helpful in diagnosis but is not pathognomonic. A higher percentage of smear cells (≥30% of lymphocytes) has been found to correlate with hypermutated *IGVH* genes (see below) and a better prognosis [23]. The presence of up to 10% of prolymphocytes (see below for description) is compatible with the diagnosis of CLL. Sometimes the administration of chemotherapy is followed by the disappearance of many of the small lymphocytes with the prolymphocytes being relatively unaffected and thus forming a higher proportion of neoplastic cells. Some cases also show a minor population of lymphoplasmacytoid lymphocytes or cells with cleft nuclei; their presence correlates with a worse prognosis [21]. In a rare morphological variant, binucleate lymphocytes comprise a significant minority of cells [24].

In the early stages of the disease the peripheral blood abnormality is confined to the lymphocytes. Later in the disease course there is a normocytic, normochromic anaemia and thrombocytopenia. Neutropenia is uncommon unless cytotoxic therapy has been administered. In CLL, both the lymphocyte count and its doubling time (greater or less

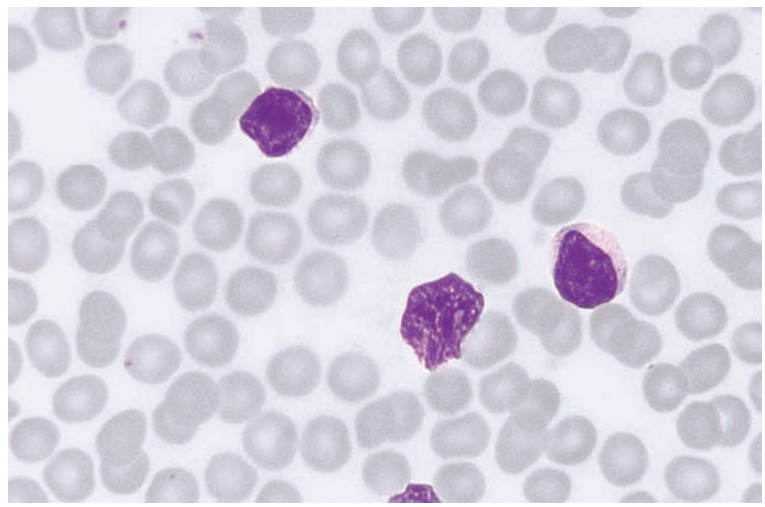


Fig. 6.3 Peripheral blood (PB) film in chronic lymphocytic leukaemia (CLL) showing two mature lymphocytes and one smear cell. Nuclear chromatin is condensed and each cell contains a barely detectable nucleolus. May–Grünwald–Giemsa (MGG) ×100.

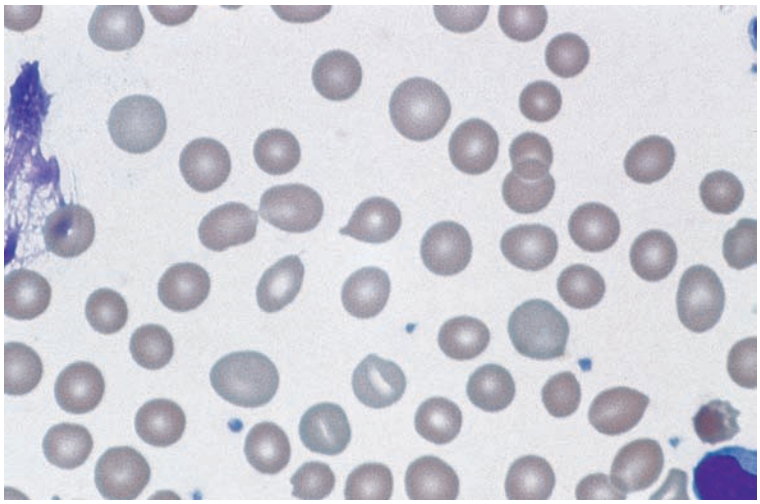


Fig. 6.4 PB film in CLL complicated by autoimmune haemolytic anaemia showing one mature lymphocyte, one smear cell, spherocytes and polychromatic macrocytes. MGG ×100.

than a year) are of prognostic significance [25]. It is important, when performing a manual differential count, to count smear cells with lymphocytes; otherwise the absolute lymphocyte count will be wrong and estimates of the doubling time will be inaccurate. Following splenectomy for cytopenia there is usually a rise in the lymphocyte count [26]. Patients with CLL have a significant incidence of autoimmune disorders affecting haemopoietic lineages. Autoimmune haemolytic anaemia (AIHA) is particularly a feature of advanced stage disease; the blood film shows spherocytes and polychromatic macrocytes (Fig. 6.4). Either the development of AIHA or the presence of a positive direct antiglobulin test is independently predictive of a worse survival [27]. Autoimmune thrombocytopenia is associated more with earlier stages of the disease, occurring at a median of 13 months from diagnosis in one study [28], and may be the presenting feature [29]. The blood count and film show reduction of platelet numbers out of proportion to the degree of anaemia. The development of autoimmune thrombocytopenic purpura is an independent adverse prognostic factor [28]. Occasional patients develop Evans syndrome, i.e. AIHA plus autoimmune thrombocytopenia. The incidence of both autoimmune thrombocytopenia and of AIHA has been reported to be increased by fludarabine therapy but this association has been disputed. In a large prospective study a positive direct antiglobulin test was similarly frequent with chlorambucil alone and fludarabine alone and was least frequent with fludarabine plus cyclophosphamide [27]. Pure red cell aplasia is a less recognized association of CLL, which may be under-reported [29]. It usually presents with anaemia of sudden onset and, if anaemia itself is excluded from the criteria for staging, it is associated mainly with early stage disease. The blood film shows normocytic, normochromic red cells with an inappropriate lack of polychromasia and a reticulocyte count of close to zero. Severe anaemia and reticulocytopenia with preservation of a normal platelet count suggest this diagnosis. Autoimmune neutropenia occurs in about 1% of patients [30]. One case has been reported in association with fludarabine therapy [31]. Automated full blood counters produce characteristic scatterplots and histograms in patients with CLL (Figs 6.5 and 6.6).

The bone marrow aspirate is hypercellular as a consequence of infiltration by lymphocytes with similar features to those in the peripheral blood. The bone marrow lymphocyte percentage has been found to be of prognostic significance in some series of patients. In AIHA the bone marrow shows erythroid hyperplasia while in pure red cell aplasia there is a striking reduction in red cell precursors. In autoimmune thrombocytopenia, the bone marrow aspirate shows normal numbers of megakaryocytes. The bone marrow may show increased mast cells

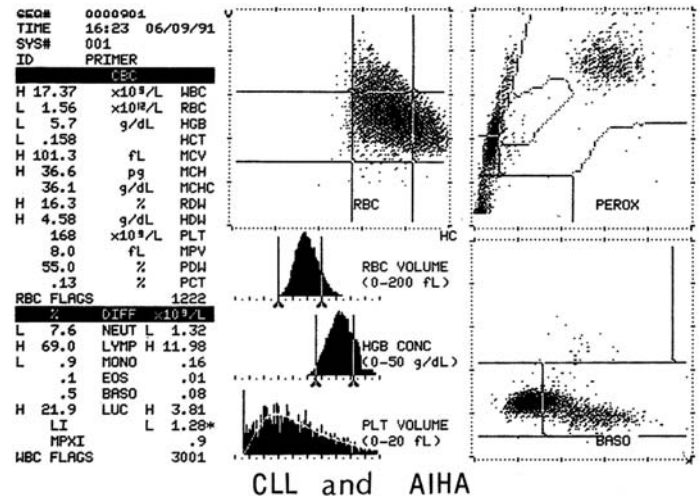


Fig. 6.5 Histograms and scatterplots on a Bayer-Technicon H2 automated counter of peripheral blood from a patient with CLL complicated by autoimmune haemolytic anaemia. In the peroxidase channel scatterplot the neoplastic lymphocytes form a fusiform cluster extending from the 'debris' area (bottom left) through the lymphocyte box to the LUC box. (LUC = large unstained, i.e. peroxidase-negative, cells.) The presence of spherocytes has led to an increased proportion of hyperdense cells.

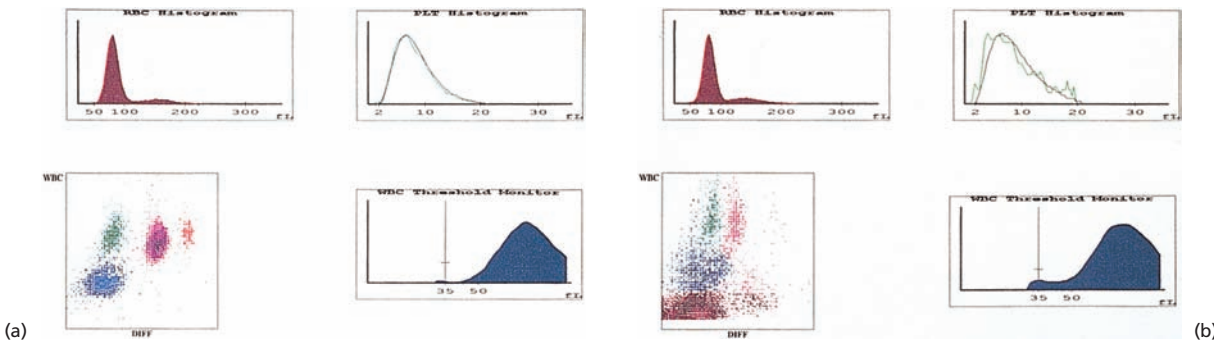


Fig. 6.6 Scatterplots on a Coulter Electronics Gen-S automated counter of PB from a patient with CLL (a) in comparison with the blood of a normal volunteer (b). The CLL sample shows an expanded lymphocyte cluster.

(Fig. 6.7). Bone marrow aspiration and trephine biopsy are not necessary for diagnosis of CLL but are recommended in the investigation of cytopenia and before initiation of therapy [20]. They should also be performed if the immunophenotype is not totally typical and the diagnosis could therefore be in doubt, e.g. if the immunophenotype score (see below) is 3 or less. CLL may undergo a prolymphocytoid transformation (see below) or a large cell transformation, referred to as Richter syndrome. In prolymphocytoid transformation both the blood and the bone marrow show prolymphocytes, whereas in Richter syndrome the site of transformation is usually a lymph node or other tissue and the peripheral blood and bone marrow may show only the features of CLL. In other patients with Richter syndrome, transformed cells are present in the peripheral blood and bone marrow (Fig. 6.8). The proliferation fraction may be high (Fig. 6.9). Richter syndrome represents transformation of a cell of the original clone in only two thirds of patients. When the two B-cell neoplasms are of independent clonal origin the emergence of the large cell lymphoma may be the result of immune deficiency with advanced disease. Epstein–Barr virus (EBV) has been implicated in Richter syndrome, both in cases showing and not showing clonal identity with the CLL cells [32,33]. Fludarabine is suspected of being an aetiological factor [33]. Uncommon transformations are to Burkitt lymphoma or Hodgkin lymphoma. In some cases of Hodgkin lymphoma the Reed–Sternberg

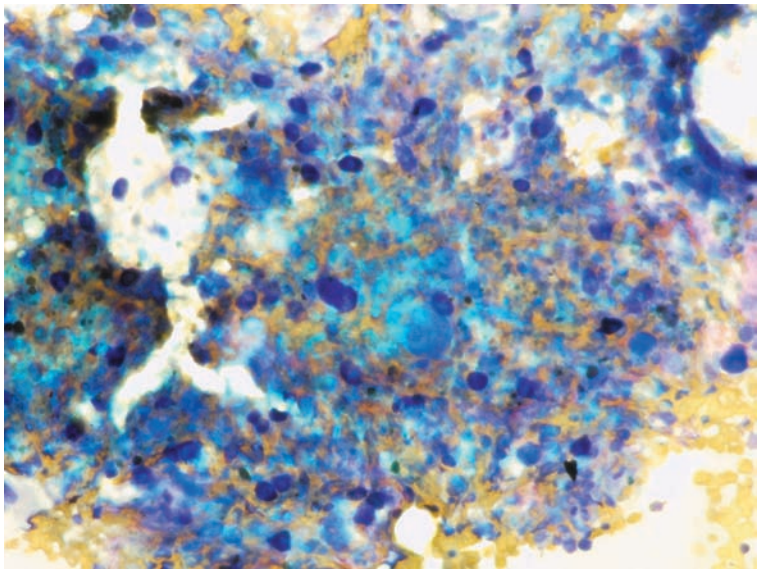
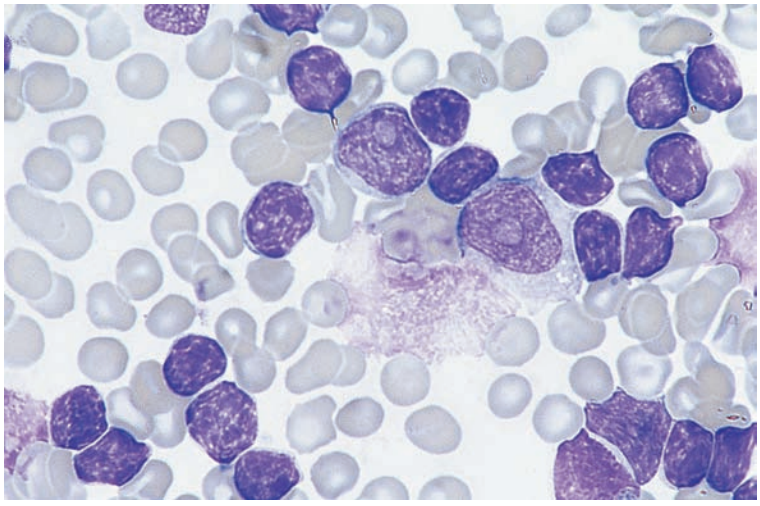


Fig. 6.7 Bone marrow aspirate in CLL showing a fragment containing numerous dispersed mast cells, which are stained deep purple. MGG ×40.

(a)



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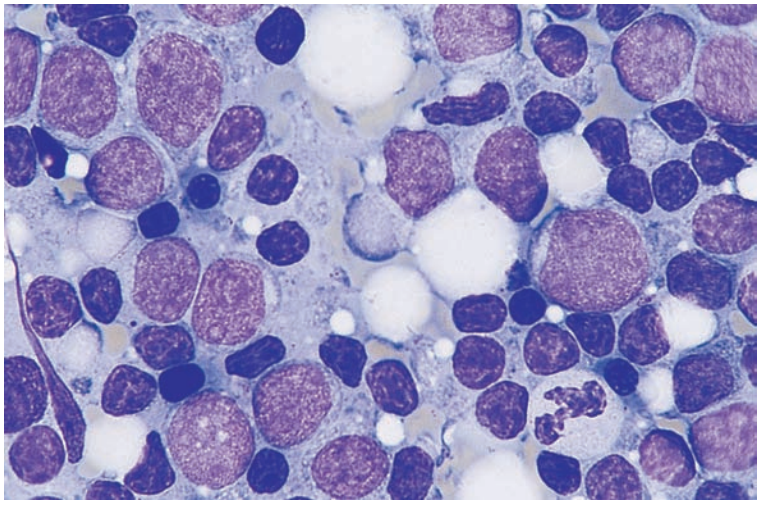

Fig. 6.8 (a) PB film in Richter syndrome showing residual mature small lymphocytes and two large, immunoblast-like cells. (b) BM film in Richter syndrome (same patient as in (a)) showing a mixture of residual small mature lymphocytes and large, immunoblast-like cells. MGG ×100.

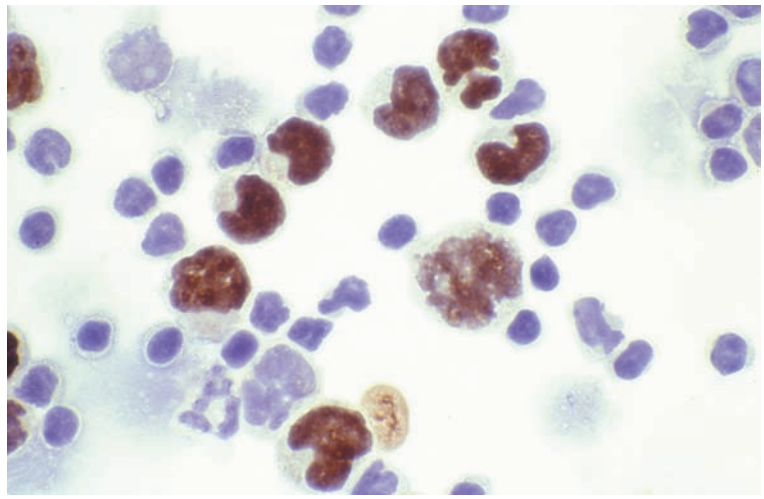

Fig. 6.9 Immunocytochemistry showing a high proliferative fraction in Richter syndrome; two of the large lymphoid cells are negative for the Ki-67 antigen and eight show strong nuclear staining. Immunocytochemistry for Ki-67.

and Hodgkin cells appear to be derived from the CLL clone [33].

The Rai [34] and Binet [35] staging systems for CLL incorporate clinical and haematological features (Tables 6.9 and 6.10). It is also possible to combine these two systems (see footnote to Table 6.10). In addition, it is useful to recognize patients with 'smouldering chronic lymphocytic leukaemia'

Table 6.9 The Rai staging system for chronic lymphocytic leukaemia [34].

Stage	Prognosis	Criteria
0	Favourable	Peripheral blood and bone marrow lymphocytosis only
I	Intermediate	Lymphocytosis and lymphadenopathy
II	Intermediate	Lymphocytosis plus hepatomegaly, splenomegaly or both
III	Unfavourable	Lymphocytosis and anaemia (Hb less than 110 g/l)*
IV	Unfavourable	Lymphocytosis and thrombocytopenia (platelet count less than 100×10^9/l)*

Hb, haemoglobin concentration.
* Anaemia or thrombocytopenia with an immune basis also lead to categorization as stage III or IV disease [20].

Table 6.10 The Binet staging system for chronic lymphocytic leukaemia [35].*

Stage	Prognosis	Criteria
A	Favourable	Lymphocytosis with no more than two regions† having enlargement of lymph nodes or other lymphoid organ; Hb greater than 100 g/l and platelet count greater than 100×10^9/l
B	Intermediate	Lymphocytosis with enlargement of lymph nodes or other lymphoid organ in three or more regions; Hb greater than 100 g/l and platelet count greater than 100×10^9/l‡
C	Unfavourable	Hb less than 100 g/l, platelet count less than 100×10^9/l or both‡

Hb, haemoglobin concentration.
* It is also possible to combine the Rai and Binet staging systems, giving the following stages: A(0), A(I), A(II), B(I), B(II), C(III), C(IV).
† A region being cervical (including Waldeyer's ring), axillary, inguinal (including femoral), liver or spleen.
‡ Anaemia or thrombocytopenia with an immune basis also lead to categorization as stage B or C disease [20].

Table 6.11 Criteria for a diagnosis of smouldering chronic lymphocytic leukaemia.

- Binet stage A
- Non-diffuse pattern of bone marrow infiltration
- Haemoglobin concentration greater than 130 g/l
- Lymphocyte count less than 30×10^9/l
- Lymphocyte doubling time greater than 12 months

(Table 6.11) in whom the disease is likely to run a very indolent course. UK Leukaemia Research Fund trials have shown that progressive stage A disease (e.g. lymphocyte doubling time less than 12 months, falling haemoglobin concentration (Hb) and falling platelet count) has the same survival as stage B disease and should therefore be grouped with it.

Immunophenotype

The cells of CLL express SmIg weakly; expression is usually no more than 1 log greater than the negative control and sometimes no expression is detected. The heavy chain most often expressed is immunoglobulin (Ig) M, with or without IgD. Cells are positive for B-cell-associated antigens such as CD19, CD20, CD24 and human leucocyte antigen DR (HLA-DR) (see Table 6.8). In addition, leukaemic cells of the great majority of patients express CD5 (also expressed by T cells) (Fig. 6.10) and CD23 [36]. Expression of CD20 is usually weak although bone marrow and lymph node cells show stronger expression than circulating cells [37]. Reactivity with FMC7 and expression of CD22 and CD79b are usually absent or weak [38]; in three series of patients, CD79b was reported to be negative in 84%, 85% and 95% of cases respectively [39]. Surface membrane CD79a expression is reduced compared with normal B cells [40], the molecule being retained in the endoplasmic reticulum. The complement receptor C3bR (CD35), which is expressed on normal B cells, is usually absent from CLL cells [41]. In most series of patients, CD11c and CD25 have generally been found to be expressed weakly, if at all, although in one series of patients 41% of patients were found to express CD25 in more than 30% of cells and CD11c has been reported as positive in between 13% and 70% of patients in different series [42,43]; when positive, expression is weaker than in hairy cell

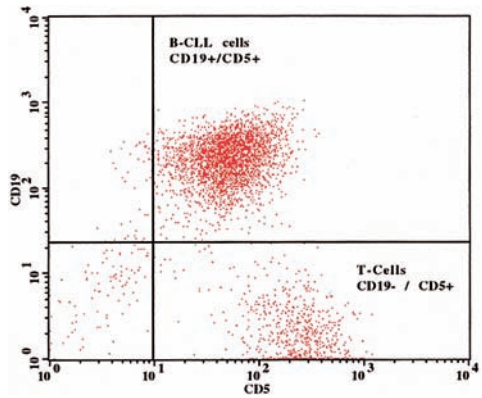

Fig. 6.10 Immunophenotyping by flow cytometry in CLL using a CD19 monoclonal antibody (McAb) labelled with phycoerythrin and a CD5 McAb labelled with fluorescein isothiocyanate; there is a major population of CD19+ CD5+ CLL cells and a minor population of CD19– CD5+ normal T cells. (By courtesy of Mr Ricardo Morilla, London.)

leukaemia [43]. CD21, the C3d complement receptor and EBV receptor, is more weakly expressed than on normal B cells [41]. CD1c is expressed on about half of normal B cells, but is less often expressed in CLL [44]. CD45 is also often expressed more weakly than in other lymphoproliferative disorders and more weakly than by normal B and T lymphocytes. CD45RA is expressed. CD43 (used in immunohistochemistry) is expressed [45,46]; this marker may be useful in making a distinction from NHL, although mantle cell lymphoma is also positive. CD40 is expressed and CD37 is strongly expressed. CD38 is expressed in 40–50% of cases and indicates a worse prognosis. CD69 is expressed in about half of patients [47]. Cells of CLL show weak or moderate cytoplasmic or membrane expression of CD138, an antigen that is typically expressed by plasma cells and lymphoplasmacytoid cells [48]. Strength of expression of CD52 is similar to that of normal lymphocytes [49], a fact of relevance to the therapeutic use of McAb with this specificity. There is expression of CD27, which is expressed on about 40% of normal circulating B cells. CD71 is expressed. CD160 is aberrantly expressed, otherwise being expressed by hairy cells and subsets of T and NK cells [50]. CD200 is more strongly expressed than by normal B lymphocytes or the cells of other lymphoproliferative disorders

[51]; quite weak expression is found in mantle cell lymphoma, which is potentially useful when there is diagnostic difficulty. Overexpression of *BCL2* protein, in comparison with expression on normal B lymphocytes, can be demonstrated [45]. Nuclear cyclin D1 is expressed in a significant minority of patients [52]. Nuclear expression of Ki-67, a marker of proliferating cells, is very variable [53]. Aberrant expression of myeloid or T-lymphocyte markers is quite common, being observed in 34% of patients in one study: CD2 was observed in 17%, CD13 in 17%, CD7 in 3.4% and CD33 in 0.9% [54]. In the same study CD10 was expressed in 10% of patients and unexpected expression of CD34 (without expression of TdT) was observed in 7.7% [54]. CD8 is expressed in a small minority of patients, 2% in one study [55,56].

A worse prognosis in CLL has been linked to expression of both IgM and IgD rather than expression of IgD alone [57], expression of CD25 [58,59], FMC7 [58], CD69 [47] and CD14 [58], high levels of expression of Ki-67 [53] and expression of cyclin D1 in more than 5% of cells [52]. Aberrant expression of myeloid or T-lymphocyte markers is also associated with a worse prognosis [54]. However, the most important immunophenotypic indicators of poor prognosis are ZAP70 expression [60,61] and CD38 expression [62–68]. Since CD38 and ZAP70 are expressed strongly by T cells and NK cells, it is important that measurements are made on CD5+ CD19+ B cells. Detection of ZAP70 expression requires, in addition, permeabilization of cells. CD38 expression correlates with ZAP70 expression, expression of both IgD and IgM rather than of IgD alone [57], lack of somatic hypermutation of *IGVH* (see below) [67], larger cells [69] and the development of autoimmune complications [67]. CD38 may be expressed on all cells or on a subpopulation of cells; either pattern of expression is associated with a poor prognosis [67]. ZAP70 expression similarly correlates with lack of somatic hypermutation and also with CD38 expression. The major factors indicating prognosis in CLL are summarized in Table 6.12 [70–77].

The diagnosis of CLL is greatly aided by use of a scoring system that incorporates those immunophenotypic markers giving the best discrimination between CLL and NHL (Table 6.13). Cases showing expression of CD5 but not CD23 can be confused with mantle cell lymphoma; features that help to make the distinction include the weak SmIg in CLL and the difference in the strength of expression of CD20 and CD19. In mantle cell lymphoma CD19 is more weakly expressed than CD20 whereas the reverse is true in CLL [78].

In patients with early CLL, confirmation of clonality by demonstration of light-chain restriction can be facilitated by analysing κ and λ expression only on CD5-positive B cells.

Immunophenotyping of peripheral blood cells shows that absolute numbers of T cells, particularly CD8-positive T cells, are increased.

With the availability of new therapeutic modalities it is now feasible to seek to eliminate detectable disease with the hope of improving survival. Immunophenotyping can be used for the detection of MRD. In this context, detection of CD19+ CD5+ cells may not be sufficient since reactive cells with this immunophenotype may appear after stem cell transplantation [79]. In addition, normal individuals have, on average, about 12% of CD5-positive B cells in the peripheral blood [80]; although these normal B cells generally express CD5 more weakly than do CLL B cells, there are a significant minority of haematologically normal subjects who have a population of strongly CD5-expressing B cells. Monitoring MRD is therefore done more effectively using four-colour flow cytometry and various combinations of antibodies (e.g. CD19+ CD5+ and either κ+ or λ+ or, alternatively, CD19+ CD5+ CD20+ CD79b–) [81].

In Richter syndrome the immunophenotype of CLL is generally retained although there may be altered expression of one or more antigens [32]. For example, CD5 expression may be lost. It seems likely that whether or not the immunophenotype is that of CLL is related to whether or not there is a large cell transformation of the original clone or a large cell lymphoma with an independent clonal origin.

Histology

Trephine biopsy histology shows a pattern of infiltration which is either interstitial, nodular, mixed nodular and interstitial, or diffuse. The pattern of infiltration correlates with the stage of the disease, with interstitial infiltration being commonest in the earliest stages of the disease and a packed

Table 6.12 Factors indicating a worse prognosis in chronic lymphocytic leukaemia.

Biological features
Male gender*
Advanced stage disease
Peripheral blood and bone marrow features
Smear cells less than 30%
Doubling time of lymphocyte count less than 12 months or lymphocyte count greater than 30×10^9/l in Binet stage A patients
Prolymphocytes in blood
Bone marrow showing diffuse infiltration ('packed marrow' pattern)
Higher soluble CD23 or CD138 in serum
Higher β2 microglobulin
Higher lactate dehydrogenase
Higher serum thymidine kinase
Higher plasma thrombopoietin [70]
Enhanced bone marrow angiogenesis and higher blood VEGF levels [71]
More numerous mast cells [72]
Features of leukaemic cells
Higher ZAP70 expression (e.g. greater than 20% of cells)† or higher CD38 expression (e.g. greater than 30% of cells)† or both
Higher expression (>45% of cells) of CD49d [73]
Expression of lipoprotein lipase
Expression of CLLU1 (CLL upregulated 1)
Unmutated *IGVH*†‡ or use of VH3.21 whether *IGVH* is hypermutated or not [74]
Del(17)(p13) in comparison with del(6)(q21) or del(13)(q14) or no abnormality detected; del(11)(q23) and trisomy 12 are intermediate
TP53 or *ATM* loss or mutation
–938C→A polymorphism in promoter of *BCL2* gene [75]

CLL, chronic lymphocytic leukaemia; VEGF, vascular endothelial growth factor; ZAP70, zeta-associated protein 70.
* Men are more likely than women to have the prognostically unfavourable unmutated *IGHV*.
† Inter-related but independent prognostic factors; ZAP70 expression also correlates with higher stage disease, higher β2 microglobulin and more rapid lymphocyte doubling time [76].
‡ Unmutated is often defined as >98% homology with germ-line; such cases have a worse prognosis than cases showing 97–97% homology with germ-line which, in turn, has a worse prognosis than cases with <97% homology [77].

Table 6.13 A scoring system for the immunophenotypic diagnosis of chronic lymphocytic leukaemia (CLL) [36,38].

Score 1 for each of the following:
• Weak expression of SmIg
• Expression of CD5
• Expression of CD23
• No expression of FMC7
• No expression of CD79b*
A score of ≥4 points is confirmatory of CLL

*Or alternatively CD22 negative but discrimination is better if CD79b is used [38].

marrow pattern more characteristic of the later stages. However, the pattern of infiltration is also of prognostic significance, independent of stage. Diffuse infiltration ('packed marrow') is indicative of a worse prognosis than nodular or interstitial infiltration. The bone marrow, like lymph nodes, may show proliferation centres, a useful feature in making the distinction from NHL.

Lymph node biopsy features are identical to those of small lymphocytic lymphoma; there is diffuse replacement by mature small lymphocytes with indistinct proliferation centres, containing larger nucleolated cells with the cytological features of prolymphocytes or paraimmunoblasts, which may give a pseudo-follicular pattern. The spleen shows

variable infiltration of red and white pulp; white pulp infiltration usually predominates [14]. The infiltrate in the white pulp may show a pseudofollicular pattern, attributable to the presence of proliferation centres [14].

On immunohistochemistry, CD5 expression is not always detected, even when it is detected by flow cytometry. CD43 is positive. CD38 and ZAP70 expression can be detected. Cyclin D1 is not expressed, except sometimes in proliferation centres [14].

Cytogenetic and molecular genetic features

Cytogenetic abnormalities [14,66,82,83] appear to be secondary events in the development of CLL, sometimes being observed only in a subclone; sometimes different subclones have different cytogenetic abnormalities. Chromosomal abnormalities can be detected by cytogenetic analysis, by FISH (Fig. 6.11) or by whole genome scanning. FISH techniques are more sensitive than conventional cytogenetic analysis and, in CLL, are generally considered the techniques of choice. However, use of novel B-cell mitogens improves the success rate of metaphase cytogenetic analysis and permits the detection of a variety of balanced and unbalanced translocations in about a third of cases, their presence being associated with worse prognosis [84].

The most characteristic abnormalities are deletion or rearrangements with a 13q14.3 breakpoint (50–60% of cases) and del(11)(q22-23) (20% of cases). Less common are trisomy 12 (10–20% of cases), del(17)(p13) (10% of cases), del(6)(q21) (5–10% of cases), translocations with a 14q32 breakpoint (4–9% of cases) and other translocations. Some patients have complex karyotypic abnormalities. There is a negative correlation between the presence of trisomy 12 and the presence of 13q14.3 abnormalities suggesting that these karyotypic abnormalities are associated with two independent leukaemogenic mechanisms. In some studies, trisomy 12 has been found to correlate with mixed cell morphology (see below) and a worse prognosis [85,86], while isolated 13q14.3 abnormalities were seen in cases with typical morphology and a better prognosis. In other studies, poor prognosis appeared to be related to atypical morphology or to other chromosomal abnormalities rather than to trisomy 12 in isolation [25,87,88]. Del(6)(q21) is associated with high white cell counts, bulky lymphadenopathy and an intermediate prognosis, similar to that of trisomy 12. Deletion of 11q23 correlates with younger age, bulky lymphadenopathy, atypical cytology, advanced disease, rapid disease progression and, in younger patients, worse survival [66,89]. Del(11q), del(17p) and complex karyotypic abnormalities also correlate with a worse prognosis; in one large series of patients, del(17p) and del(11q) were found to be independent poor prognostic features [90]. Del(17p) may be associated with transformation. In the UK Leukaemia Research Fund CLL 4 trial, best prognosis

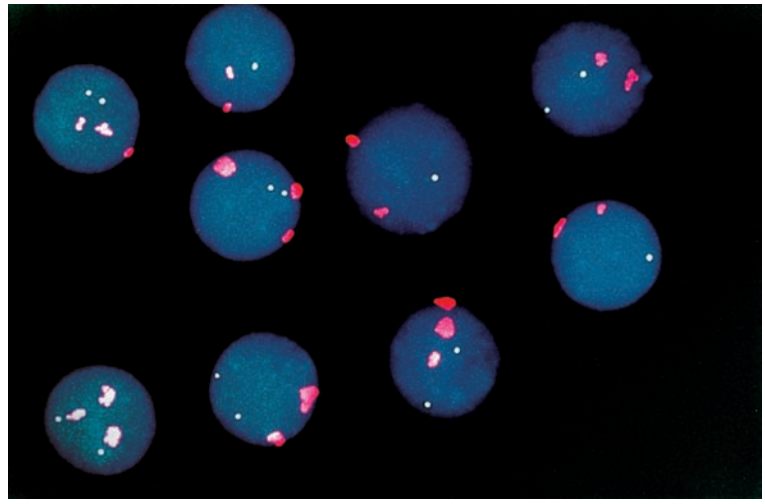

Fig. 6.11 Fluorescence *in situ* hybridization (FISH) in CLL using directly labelled probes for chromosome 12 (pink) and *RB1* (white). In this patient there were three subclones within the population of CLL cells: (a) cells that are disomic for chromosome 12 and have both alleles of *RB1* (two pink dots and two white dots); (b) cells that are disomic for chromosome 12 but have a deletion of one allele of *RB1* (two pink dots and one white dot); (c) cells that have trisomy 12 and no *RB1* deletion (three pink dots and two white dots). (By courtesy of Dr J. Garcia Marco, Cambridge.)

was associated with del(13q) and absence of detectable abnormality, an intermediate prognosis with trisomy 12, del(6q) and del(11q), and a considerably worse prognosis with the presence of del(17p) in more than 20% of cells; not all of these were significant [90a]. Del(11q) has been reported to be associated with a lower response rate than the other intermediate group abnormalities but with the survival being much the same. A small minority of patients (1–2%) have rearrangements of *BCL2* at 18q21 with a heavy or light chain locus. In comparison with follicular lymphoma, t(14;18)(q32;q21) is less common while t(2;18)(p12;q21) and t(18;22)(q21;q11) are more common [82]; the breakpoints on chromosome 18 differ at a molecular level from the breakpoints in follicular lymphoma [91]. A t(14;19)(q32;q13) translocation is found in less than 1% of patients, the genes involved being *IGH* and *BCL3* [92]; these patients have atypical features including relatively young age and atypical cytology and immunophenotype [93]. Rearrangements at 14q32 are also associated with a poor prognosis [94]. *MYC* translocation associated with t(8;14)(q24.1;q32), t(8;22)(q24.1;q11) or t(2;8)(p12;q24.1) is associated with increased prolymphocytes and poor prognosis [95]. t(11;14)(q13;q32) and *BCL1* rearrangement have been reported in a small proportion of cases of CLL but it is quite likely that this represents misdiagnosis of mantle cell lymphoma.

Some correlation between karyotype and immunophenotype has been observed. Cases with trisomy 12 are more likely to express FMC7 and show strong expression of SmIg [96]. They are more likely to have strong expression of CD20 and to respond to rituximab [97]. Cases with a complex karyotype are also more likely to express FMC7 [96]. Cases with del(11q) have been found to have reduced expression of numerous adhesion molecules, including CD11a/CD18, CD11c/CD18, CD48 and CD58 [98]. Expression of CD20 is also low and may indicate lower probability of response to rituximab [97].

Molecular genetic analysis has shown that CLL can arise either from a mutation in a B cell with non-mutated *IGVH* genes or from a post-germinal centre memory B cell with hypermutated *IGVH* genes. The former has a worse prognosis with a median survival of about 3 years whereas it is about 7 years in those with hypermutated genes [14,65,99,100]. The differences observed in these two subsets of CLL are summarized in Table 6.14. Origin from a cell with non-mutated *IGVH* genes has been reported to correlate well with CD38 expression [63,67,106] although some observers have reported poorer correlation [62,65,107]. Unmutated genes and CD38 expression have been found to be independent poor prognostic features [107]. ZAP70 expression also correlates with unmutated genes and indicates a worse prognosis [60]. In one study ZAP70 was a better surrogate marker for unmutated genes than CD38 [60] and in another it gave more prognostic information than either CD38 expression or mutational status; on multivariate analysis, mutational status divided the ZAP70-negative cases into two further prognostic groups but CD38 expression gave no further information [61]. The presence of *BCL6* mutation identifies a poorer prognosis subset within the groups of patients with hypermutation of *IGVH* [105].

At a molecular level, one study of abnormalities on chromosome 13 [108], found some patients to have deletion of the *RB1* gene at 13q14 but deletion of the *DBM* locus distal to *RB1* was more common and deletion at 13q12.3, encompassing the *BRCA2* gene, was the most frequent abnormality. Other potentially relevant deleted genes in this region are two microRNA genes, *MIRN16-1* and *MIRN15A* [14]. *ATM* at 11q23 is deleted in patients with del(11q) and overall is mutated in about 20% of patients [109]; mutations are seen particularly in patients in whom the other allele is deleted [110]. The *BCL6* gene is mutated in about a quarter of patients [111]. The leukaemogenic mutation associated with trisomy 12 has not yet been elucidated but amplification of the *MDM2* gene at 12q13-15 has been suggested as a possible mechanism [83]. Mutations of the *CD79B* gene have been reported to be common and to correlate with lack of expression of the corresponding antigen, this being considered likely to be responsible for the weak expression of SmIg in CLL [112]. However this was not confirmed in other series of patients; transcription of *CD79B* was found to be only slightly reduced with a post-transcriptional defect, specifically a failure of assembly of the B-cell receptor complex, being responsible for

Table 6.14 Differences between chronic lymphocytic leukaemia subsets with unmutated and hypermutated immunoglobulin heavy chain variable region (*IGVH*) genes.

	Somatic hypermutation of *IGVH* genes absent (>98% homology with germ-line)	Somatic hypermutation of *IGVH* genes present
Epidemiology	M : F = 3 : 1	M : F = 1 : 1
Putative cell of origin	Pre-germinal centre B cell	Post-germinal centre antigen-experienced memory B cell (CD5-positive subset)
Immunophenotype	CD38 usually expressed; *MUM1-IRF4* not expressed [101]; CD180 less strongly expressed [102]; IgM more strongly expressed [102]	CD38 not usually expressed; *MUM1-IRF4* expressed [101]; CD180 more strongly expressed [102]; IgM less strongly expressed [102]
Cytogenetics	Increased prevalence of abnormalities of 11q and 17q and, in some studies, of trisomy 12 [14]	Increased prevalence of 13q14.3 abnormalities [14]
Molecular genetics	*TP53* dysfunction (due to *TP53* or *ATM* mutation) more common; *ZAP70* expression [103]; *CLLU1* markedly up-regulated [104]	*TP53* dysfunction less common; lack of *ZAP70* expression [103]; *CLLUL1* moderately up-regulated [104]; *BCL6* may be mutated and correlates with worse prognosis [105]
Histology	Proliferation centres absent in trephine biopsy sections [106]	Proliferation centres may be present in trephine biopsy sections
Prognosis	Worse	Better

the reduced surface expression of CD79b and IgM [113]. Deletion of 17p13 leads to loss of *TP53*. Deletion or mutation of *TP53*, the tumour suppressor gene that encodes p53, is relatively uncommon in typical CLL and correlates with progressive disease, refractoriness to therapy and poor prognosis [114]; overall, a *TP53* mutation or deletion is found in about 15% of patients. *TP53* deletion is usual in patients with deletions or translocations involving 17p13 and in these patients the other allele of *TP53* is usually mutated or deleted [110]. In other patients, dysfunction of p53 is the result of *ATM* mutation, probably biallelic loss or mutation [115]. Strong expression of p53 correlates with worse prognosis [116]. Mutation of *ATM* is found in about 12% of patients and correlates with refractoriness to chlorambucil and fludarabine and with worse survival, independent of *IGVH* status [117]. The prevalence of various cytogenetic abnormalities and loss of specific cancer suppressor genes can be related to whether or not *IGVH* shows hypermutation (see Table 6.14). Although *BCL2* is often overexpressed, rearrangement is demonstrable in only a small minority of patients [82].

Problems and pitfalls

The blood film of CLL is often so characteristic that diagnosis from the blood film is likely to be reliable. Nevertheless, as treatments for chronic lymphoproliferative disorders become more specific, immunophenotypic confirmation is essential in this and other related conditions. Confusion has occurred with mantle cell lymphoma, T-cell prolymphocytic leukaemia and even benign conditions such as post-splenectomy lymphocytosis and polyclonal B lymphocytosis.

Atypical chronic lymphocytic leukaemia

Atypical CLL is a chronic B-lineage lymphoproliferative disorder, which shares many features with CLL but shows cytological and sometimes immunophenotypic and cytogenetic differences.

Clinical, haematological and cytological features

The French–American–British (FAB) group defined two cytologically atypical variants of CLL, designated collectively CLL, mixed cell type [4]: in some patients there was a spectrum of cells from small to large lymphocytes (but with fewer than 10%

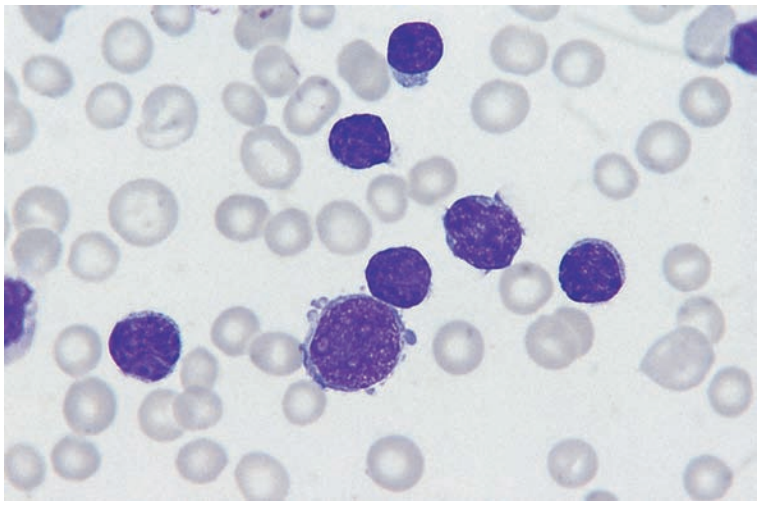

Fig. 6.12 PB film in CLL, mixed cell type, showing small mature lymphocytes and several larger cells, one with multiple nucleoli. MGG ×100.

prolymphocytes) with an associated tendency to cytoplasmic basophilia, while in others there was an increase of prolymphocytes so that they constituted more than 10% but fewer than 55% of lymphocytes (designated CLL/PL) [118,119] (Fig. 6.12). The CLL/PL group included patients who presented *de novo* with this morphology and others who underwent a 'prolymphocytoid transformation' of CLL. In one series of patients, those with CLL of mixed cell type tended to present with more advanced disease and to have a worse prognosis than in classical CLL [120]. In another series the presence of more than 10% of prolymphocytes correlated with a worse prognosis [25]. Others have defined other atypical forms of CLL including cases with lobulated nuclei, correlating with trisomy 12 and worse prognosis [25], and cases with at least 10% of cells with deeply cleft or lobulated lymphocytes, a group associated with a higher white blood cell count (WBC), a lower platelet count and a worse prognosis [121].

Immunophenotype

About two thirds of cases of CLL of mixed cell type have an immunophenotype typical of CLL. The other third show atypical features such as strong expression of SmIg or CD20 or expression of CD11c or FMC7. In one study, atypical CLL with cleft or lobulated lymphocytes was associated with stronger expression of CD23 [121]. Atypical morphology is more common when there is expression of both IgD and IgM than when only IgD is expressed [57].

Cytogenetic and molecular genetic features

Trisomy 12 is commoner in cases of atypical CLL than in CLL with typical cytological features, whereas a normal karyotype and del(13q) are less common [108]. *TP53* abnormalities are much more common than in typical CLL and are likely to be associated with progression of CLL to CLL/PL [114]. The development of trisomy 12 and of *TP53* abnormalities may represent independent pathways of transformation from CLL to CLL/PL [114].

Patients whose cells have atypical morphology (more than 10% prolymphocytes or more than 15% cleaved or lymphoplasmacytoid cells) are more likely to have a clonal origin from a non-mutated B cell, a molecular genetic feature associated with a worse prognosis [100].

Problems and pitfalls

By definition, the blood film of atypical CLL differs from that of typical CLL. It can be confused with that of NHL, particularly mantle cell lymphoma. The diagnosis requires consideration of both the cytological features and the immunophenotype.

Monoclonal B-cell lymphocytosis

A small but significant percentage of reasonably healthy adults who are apparently haematologically

normal can be demonstrated to have increased numbers of monoclonal B lymphocytes (with a clonal rearrangement of *IGVH* genes) in the peripheral blood [3,122,123]. B-cell numbers exceed the upper limit of normal of 0.49×10^9/l. The total lymphocyte count may be normal or increased but the criteria for a diagnosis of CLL are not met. In one survey of hospital outpatients aged between 62 and 80 years, the prevalence of monoclonal B-cell lymphocytosis with a normal total lymphocyte count was 6.8% [123]. In about three quarters of such cases the immunophenotype resembled that of CLL and in the other quarter, that of NHL [123]. Clonal cytogenetic abnormalities typical of CLL were often present, 13q14 abnormalities in 39% and trisomy 12 in 18%; interestingly the poor prognosis abnormalities, del(11)(q23) and del(17p), were not found [123]. Somatic *IGVH* hypermutation, a good prognosis feature in CLL, was present in 88% [123] and CD38 expression was usually low, also a marker of indolent CLL. Some patients with monoclonal B-cell lymphocytosis progress to CLL but how often this happens is not clear. At median follow-up of 6.7 years, clinically progressive CLL occurred in 15% of the patients with an initial lymphocyte count of more than 4×10^9/l with another 13% showing a continuing rise in the lymphocyte count; follow-up was not done in patients with an initially normal blood count [123]. Patients who present with CLL have usually had preceding monoclonal B-cell lymphocytosis [15].

Suggested diagnostic criteria are shown in Table 6.15 [124].

The prevalence of monoclonal B-cell lymphocytosis is higher (about 13%) in first-degree relatives of patients with CLL [125].

Table 6.15 Criteria proposed for the diagnosis of monoclonal B-cell lymphocytosis [124].

- Evidence for B-cell clone:
  There is a κ : λ ratio of >3 : 1 or <0.3 : 1
  or
  There are more than 25% of B cells with absent or low level surface membrane immunoglobulin
  or
  There is a disease-specific immunophenotype
- Abnormality persists for 3 months
- There is no lymphadenopathy, hepatomegaly or splenomegaly or autoimmune or infectious disease; the absolute count of B lymphocytes does not exceed 5×10^9/l and there are no features diagnostic of a B-lineage lymphoproliferative disorder (other than possibly a paraprotein)

B-cell prolymphocytic leukaemia

B-cell prolymphocytic leukaemia (PLL) is a rare chronic B-lineage lymphoproliferative disorder with prominent splenic involvement, specific cytological features and an immunophenotype resembling that of NHL rather than CLL [126].

Clinical, haematological and cytological features

There is a higher median age of onset than in CLL. Characteristically there is a high WBC and marked splenomegaly with only trivial lymphadenopathy [127].

The predominant cell is the prolymphocyte (Figs 6.13 and 6.14); a cut-off point of 55% of such cells has been found to be most useful in separating prolymphocytic leukaemia from CLL/PL [119]. The prolymphocyte is a large cell with often relatively abundant cytoplasm. The nucleus is round with relatively well-condensed nuclear chromatin and with a prominent vesicular nucleolus showing perinucleolar chromatin condensation. One patient whose cells showed erythrophagocytic activity has been described [128].

Anaemia and a high lymphocyte count have been found to correlate with a worse prognosis [129]. A positive direct antiglobulin test and AIHA may occur [129]. There may also be thrombocytopenia. The serum immunoglobulin concentration is often reduced and a paraprotein is present in about a third of patients [129].

Immunophenotype

About two thirds of cases of PLL have an immunophenotype that differs markedly from that of CLL. SmIg is strong, CD5 expression is weak and FMC7 and CD20 expression are also strong. In the other third of cases, the immunophenotype is intermediate between the typical CLL phenotype and the typical PLL phenotype. In some patients, leukaemic cells express CD5 but show expression of FMC7 and strong expression of SmIg, thus immunophenotypically resembling cells of mantle cell lymphoma [130]. In the majority of cases of PLL there is expression of IgM with or without IgD but

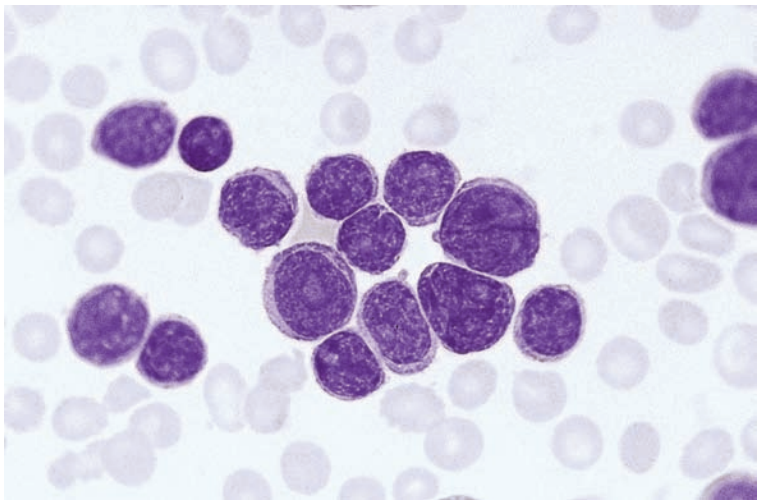

Fig. 6.13 PB film in B prolymphocytic leukaemia showing cells that are regular in shape with round nuclei. Nuclear chromatin shows some condensation and the larger cells contain prominent vesicular nucleoli. MGG ×100.

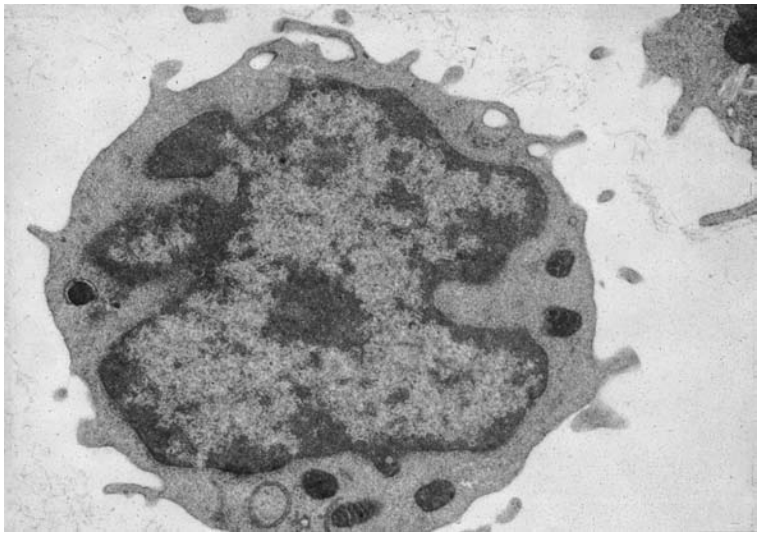

Fig. 6.14 Ultrastructural examination In B-lineage prolymphocytic leukaemia showing a prominent nucleolus and abundant cytoplasmic organelles. (By courtesy of Professor Daniel Catovsky, London.)

in a minority there is expression of IgG or IgA. In contrast to CLL, CD79b is almost always expressed [131], as is CD22 [132]. CD11c is strongly expressed in the majority of patients [43]. In one series of patients, CD10 and CD38 were often expressed [129]. ZAP70 is often expressed [126].

Histology

The bone marrow is hypercellular with lymphoid infiltration. Trephine biopsy sections show an interstitial/nodular or diffuse pattern of infiltration. Lymph node infiltration is diffuse with or without a vaguely nodular pattern. Splenic infiltration is in both the red and white pulp with large proliferative nodules in the white pulp showing a characteristic bizonal appearance, dense at the centre and lighter at the periphery [133,134].

Cytogenetic and molecular genetic features

There are often complex karyotypic abnormalities. Specific abnormalities that have been noted include trisomy 3 [132], del(6q), monosomy 7, del(7q),

del(11)(q23), del(12)(p13), del(13)(q14) and various translocations involving chromosome 14 with a 14q32 breakpoint [132,135–137]. Other translocations observed have included t(6;12)(q15;p13), t(2;3)(q35;q14) [135] and t(3;8)(p13;q13) [138]. Trisomy 12 occurs [135] but is relatively uncommon [126].

It should be noted that previously described cases associated with t(11;14)(q13;q32) are now considered to represent a leukaemic phase of mantle cell lymphoma [126].

Hypermutated and non-mutated *IGVH* genes are equally frequent but the mutational status shows no relationship to CD38 or ZAP70 expression [126]. Mutations of *TP53* [139] and deletion of *RB1* [137] and *BRCA2* occur.

Problems and pitfalls

The lack of any very definite diagnostic criteria can make the diagnosis of PLL difficult. It needs to be distinguished from atypical CLL and prolymphocytoid transformation of CLL. Distinction from mantle cell lymphoma may be difficult on cytological grounds and may thus require cytogenetic or FISH analysis. The neoplastic cells of diffuse large B-cell lymphoma in leukaemic phase tend to be more pleomorphic than those of B-PLL. T-cell PLL (see below) usually has distinctive cytological features but some cases are only distinguished by immunophenotyping.

Hairy cell leukaemia

Hairy cell leukaemia (HCL) is an uncommon chronic B-lineage lymphoproliferative disorder, usually presenting with splenomegaly and having distinctive cytological, histological and immunophenotypic features. This condition usually but not always arises in a hypermutated post-germinal centre B cell [140,141].

Clinical, haematological and cytological features

Hairy cell leukaemia occurs throughout adult life. It is four times as common in men as in women and three times as common in white Americans as in black Americans [142]. The US incidence is 0.32/100 000/year [142]. The disease is characterized by splenomegaly with little peripheral lymphadenopathy. Using computerized tomography (CT) scanning, abdominal lymphadenopathy is sometimes detected at diagnosis, mainly in patients with bulky disease. Opportunistic infections can occur and with advanced disease are common. Circulating leukaemic cells are not usually numerous and many patients are pancytopenic. Severe monocytopenia is usual. Some patients have macrocytic red cells. An Hb of less than 100 g/l, a WBC of less than 2×10^9/l and a platelet count of less than 100×10^9/l are prognostically adverse [143,144]. A minority of patients have a high WBC with more numerous circulating hairy cells. Hairy cells are larger than normal lymphocytes or CLL lymphocytes. They have moderately abundant, weakly basophilic cytoplasm with irregular 'hairy' projections and consequently an ill-defined cell outline (Fig. 6.15). The cytoplasm may contain azurophilic granules or rod-shaped inclusions. Occasionally there are parallel linear structures in the cytoplasm (Fig. 6.16) that correspond to the ribosomal lamellar complex that is demonstrated on ultrastructural examination. The nucleus is eccentric, and round, oval, dumbbell or kidney shaped. The nuclear chromatin has a finely dispersed pattern and nucleoli are inconspicuous, small and usually single. In the great majority of cases of HCL the cells show tartrate-resistant acid phosphatase (TRAP) activity (Fig. 6.17). Such activity is rare, although not unknown, in other lymphoproliferative disorders. The bone marrow is usually difficult to aspirate as a consequence of fibrosis but, when it can be aspirated, hairy cells are relatively more numerous than in the blood.

A large cell transformation may occur, most often in abdominal lymph nodes. Large cells may then be seen in the bone marrow.

Immunophenotype

Hairy cells have distinctive light-scattering characteristics on flow cytometry [130,145]; forward light scatter is usually higher than in other chronic lymphoproliferative disorders and sideways light scatter may also be high, similar to that of monocytes. Hairy cells may be missed in flow cytometric analysis if the instrument operator gates on the area where lymphocytes are normally found [78]. Nonspecific binding is common so that comparison with the negative control is important.

Hairy cells have the immunophenotype of a relatively mature B cell. B-lineage-associated antigens

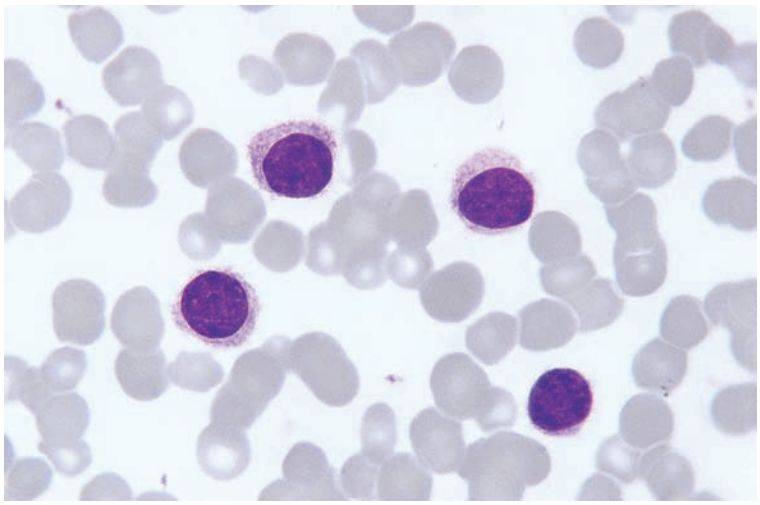

Fig. 6.15 PB film in hairy cell leukaemia. Cells have round nuclei with condensed chromatin and moderately abundant cytoplasm with ragged edges. MGG ×100.

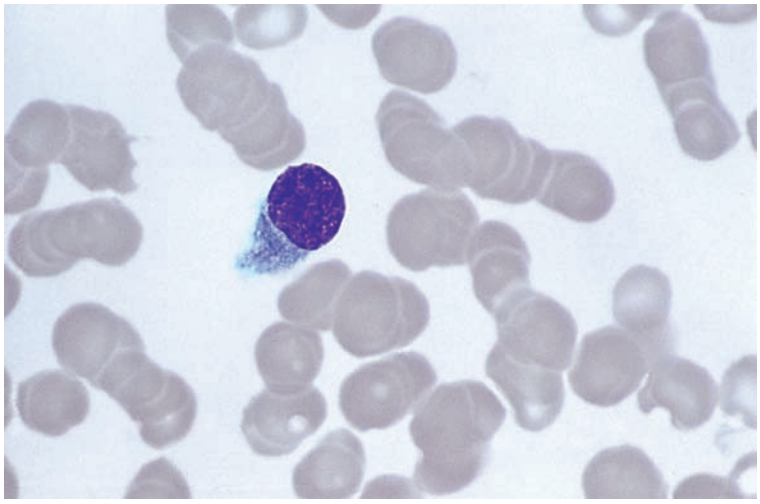

Fig. 6.16 PB film in hairy cell leukaemia showing a hairy cell containing a ribosomal lamellar complex. MGG ×100. (By courtesy of Dr Laura Sainati, Padua, and Professor Daniel Catovsky.)

CD19, CD20, CD22 and CD79a are expressed. The expression of CD20 and CD22 is strong. CD79b is positive in about a quarter of patients [131]. SmIg is moderately strongly or strongly expressed with some cases also showing cytoplasmic immunoglobulin. SmIg is IgM and sometimes also IgD, IgG or IgA. HCL is unusual among B-lineage disorders in that in 40% of cases there is expression of multiple immunoglobulin isotypes [140]. CD5, CD10, CD23 and CD43 are generally negative, with both CD10 and CD23 being expressed in about 10–15% of cases [146,147]. FMC7 is positive, as is CD25, which represents the α chain of the interleukin 2 receptor and is a marker of activated T and B cells. CD11c is usually positive and is more strongly expressed than in other lymphoproliferative disorders [78]. In addition to the expression of B-lineage-associated immunophenotypic markers, there are several markers that show a degree of specificity for hairy cells; they include CD103, CD123 and HC2 [148] (which is not commercially available). CD160 is expressed, otherwise being expressed (more weakly) by CLL cells and by subsets of T and NK cells [149]. CD27 is not expressed whereas it is usually expressed in CLL, follicular lymphoma, mantle cell lymphoma and splenic lymphoma with villous

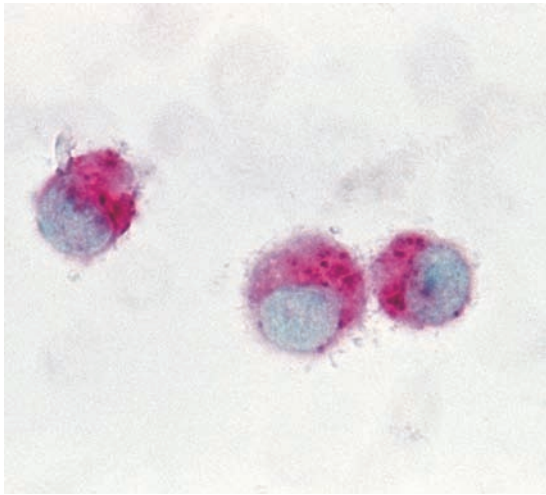

Fig. 6.17 Film prepared from a buffy coat of the PB of a patient with hairy cell leukaemia showing tartrate-resistant acid phosphatase (TRAP) activity. TRAP reaction ×100.

lymphocytes [150]. The presence of the isoenzyme of acid phosphatase identified cytochemically by TRAP activity can also be detected immunologically using a specific McAb. The use of a scoring system can help to distinguish HCL from other lymphoproliferative disorders with which it might be confused. If 1 point is scored for positivity with HC2, CD11c, CD25 and CD103 then cases of HCL almost always score 3 or 4, while cases of hairy cell variant and splenic lymphoma with villous lymphocytes score 0, 1 or 2 [151]. If HC2 is not available, CD123 can be substituted.

Flow cytometry is a very sensitive technique for the detection of hairy cells and as few as 1% of cells may be detected. The identification of small numbers of neoplastic cells is aided by a comparison of side scatter of light and CD45 expression; hairy cells appear as a discrete population of cells that express CD45 more strongly than do normal lymphocytes or NHL cells [152].

Histology

Trephine biopsy sections show infiltration that may be focal, diffuse or interstitial in a hypocellular bone marrow. There is a highly characteristic pattern of infiltration with cells appearing to be separated from each other by a clear zone (Fig. 6.18). This pattern is more apparent on paraffin-embedded specimens than in resin-embedded specimens, although the latter shows the cellular detail more clearly. The characteristic delicate chromatin pattern and indented or lobulated nuclei are usually readily apparent. Reticulin is increased. Spleen histology shows a distinctive pattern of red pulp infiltration with widening of the pulp cords and the formation of pseudo-sinuses lined by hairy cells. Immunohistochemistry on trephine biopsy sections permits the detection of expression of annexin A1 and DBA.44 (CD72) [153]. Expression of annexin A1 is highly specific as long as it is clear that it

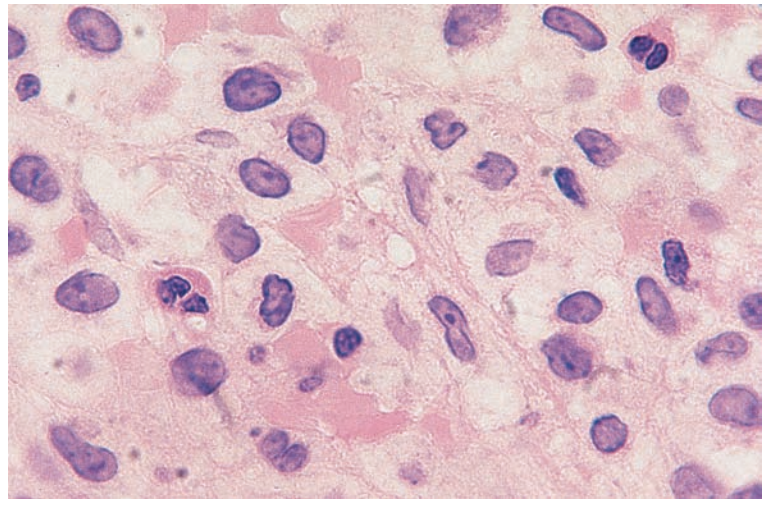

Fig. 6.18 BM trephine biopsy section in hairy cell leukaemia showing cells with round, oval or irregular nuclei and scanty, ragged cytoplasm. The spacing of the nuclei is characteristic of this disorder. Two neutrophils and one erythroblast are also present. Paraffin-embedded, haematoxylin and eosin (H&E) ×100.

is being expressed on B cells not on normal T cells or myeloid cells. Expression of CD72 is less specific. Cyclin D1 is overexpressed in 50–75% of cases without any correlation with the presence of t(11;14) or *BCL1* rearrangement [141]. Since achievement of a complete remission correlates with better survival and is therefore the aim of treatment, it is important to repeat a trephine biopsy after treatment. Immunohistochemical staining for CD20 or CD72, assessed in relation to cytological features, can help in the detection of MRD. Annexin A1 is less useful because of its expression on myeloid cells.

Cytogenetic and molecular genetic features

A great variety of cytogenetic abnormalities have been observed including trisomy 5, trisomy 6, monosomy 10, monosomy 17, monosomy or trisomy 12, del(6q) and, most frequently, translocations with a 14q32 breakpoint (giving rise to both add(14q) and del(14q)) [98,154,155].

Problems and pitfalls

The diagnosis of HCL may initially be missed if infrequent neoplastic cells in the peripheral blood film are not detected. The presence of marked monocytopenia is an indication to search for hairy cells, particularly but not only in a patient with splenomegaly. The distinction from the unrelated condition known as hairy cell variant leukaemia (see below) is on the basis of the higher WBC and the prominent nucleoli of the latter condition. In trephine biopsy sections confusion has occurred with systemic mastocytosis, because of the spacing of the nuclei, and with aplastic anaemia, when there are inconspicuous neoplastic cells in a hypocellular marrow. As long as the possibility of HCL is considered the diagnosis can be made without difficulty.

Hairy cell variant leukaemia

The 'variant' form of HCL is a rare, chronic B-lineage lymphoproliferative disorder that has no close relationship to hairy cell leukaemia although it does have some clinical and cytological similarities. In the 2008 WHO classification it is one of two provisional entities designated splenic B-cell lymphoma/leukaemia, unclassifiable [156]. Hairy cell variant differs from HCL immunophenotypically, histologically and in its responsiveness to therapy.

Clinical, haematological and cytological features

There is usually splenomegaly with little lymphadenopathy. The WBC is usually high with peripheral blood leukaemic cells being numerous [157,158]. In contrast to HCL, the monocyte count is usually normal. There may be mild anaemia and thrombocytopenia.

The neoplastic cells have a higher nucleocytoplasmic ratio than those of HCL, the cytoplasm is more basophilic and the nucleus has a more condensed chromatin pattern with a prominent nucleolus (Fig. 6.19). The nucleus resembles that of a prolymphocyte. There may be some binucleated cells and some larger cells with hyperchromatic nuclei. The TRAP reaction is almost always negative.

The distinction between HCL and hairy cell leukaemia variant is clinically important since both interferon and nucleoside analogue therapy, which are, respectively, successful and highly successful in HCL, are often ineffective in hairy cell variant.

Immunophenotype

The immunophenotype is closer to that of PLL than to that of classical HCL. B-cell associated antigens (CD19, CD20 and CD22) are expressed and FMC7 is positive. Negative reactions are usual with CD25 and HC2 although some cases are CD11c or, less often, CD103 positive. A scoring scheme for immunophenotypic markers has been found very useful if these four McAb are used [151]. CD123 is negative [138]. CD79b is positive in about a quarter to a third of patients [131]. Although the α chain of the interleukin 2 receptor (CD25) is not expressed, there is expression of the β and γ chains [138].

Histology

Trephine biopsy sections most often show an interstitial infiltrate [158]. The spaced pattern of HCL may be totally absent or there may be a mixture of areas of denser infiltrate with areas in which cells are more widely spaced. A dominant intrasinusoidal pattern of infiltration is common [138]. Reticulin is moderately increased, being less heavy than in HCL [138]. CD72 is expressed but not annexin A1. Splenic infiltration is in the red pulp with a minority of cases showing blood lakes [158].

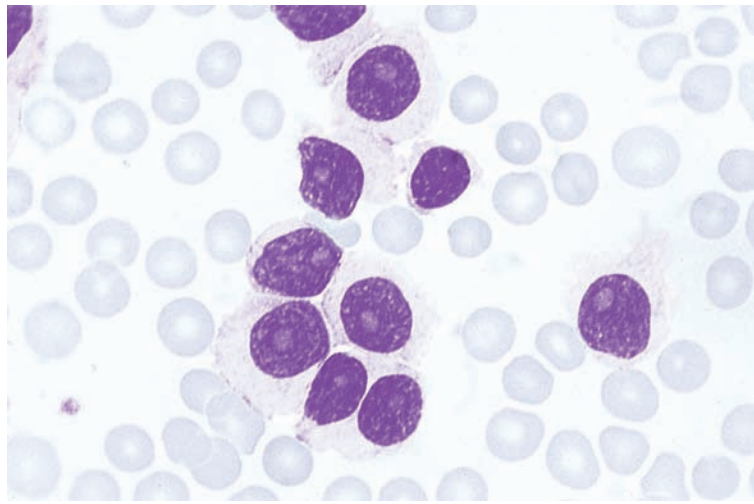

Fig. 6.19 PB film in the variant form of hairy cell leukaemia (hairy cell leukaemia variant). Cells are nucleolated and it is evident that the white cell count is high. MGG ×100.

Cytogenetic and molecular genetic features
Complex karyotypes are common as is monoallelic deletion of *TP53* [138].

Problems and pitfalls
Making a distinction from HCL is important and rests on nuclear features and immunophenotype. Splenic lymphoma with villous lymphocyte also needs to be distinguished.

Splenic marginal zone lymphoma including splenic lymphoma with villous lymphocytes

Splenic lymphoma with villous lymphocytes (SLVL), first described in 1987 [159], is a chronic B-lineage lymphoproliferative disorder, which has in the past been confused with CLL but which differs clinically, cytologically and immunophenotypically. Whereas SLVL was recognized by examination of the peripheral blood, an entity designated splenic marginal zone lymphoma (SMZL) was recognized by splenic histology [160]. In the WHO classification SLVL is recognized as a presentation of SMZL [161]. Despite its name, this lymphoma may not be derived from splenic marginal zone B cells but rather from a cell within the splenic follicular mantle [138]. The features described in different series of patients differ somewhat, depending on whether the diagnosis has been made by peripheral blood cytology or by splenic histopathology. Transformation of SLVL to a large cell lymphoma was reported in 4%, 10% and 13% in three series of patients [162] and transformation of SMZL in 13% [163]. Some patients with SMZL have an underlying hepatitis C infection, which is likely to be aetiologically relevant.

Clinical, haematological and cytological features
Splenic lymphoma with villous lymphocytes/SMZL is predominantly a splenic lymphoma with only minor lymphadenopathy [159,163–165]. The incidence rises with age and is higher in men than women.

The WBC varies from normal to moderately elevated. Up to a quarter of cases designated SLVL do not have an absolute lymphocytosis [165]. In patients with a high count the majority of circulating cells are abnormal lymphocytes, whereas in those without lymphocytosis, villous lymphocytes may be as few as 5% of cells. About a third of cases designated SMZL have no abnormal circulating cells.

The neoplastic cells are larger than CLL cells. The nucleus is round to ovoid with clumped chromatin and, in about half the cases, a distinct small nucleolus is present [4] (Fig. 6.20). The cytoplasm varies in amount, is moderately basophilic and has short villous projections, sometimes localized at one pole of the cell. The nucleocytoplasmic ratio is higher than in HCL or hairy cell variant leukaemia. A minority of cells show plasmacytoid features, i.e. the nucleus is eccentric, basophilia is more

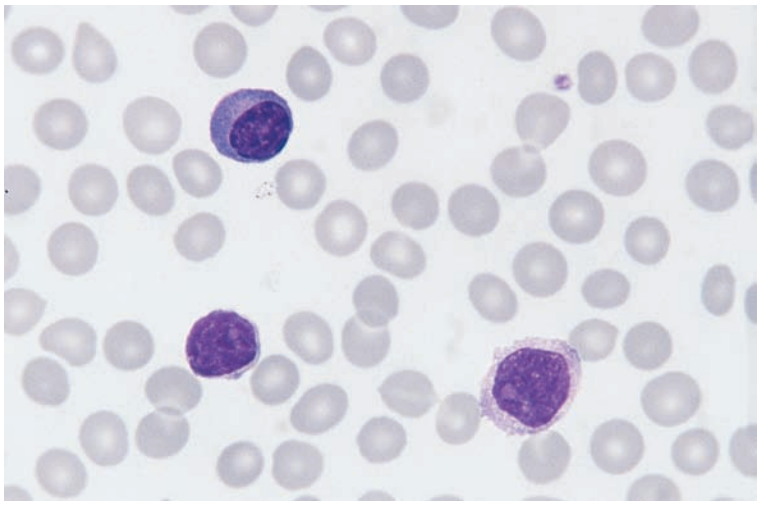

Fig. 6.20 PB film in splenic lymphoma with villous lymphocytes. The cells have small, inconspicuous nucleoli. One has villous cytoplasm and one is showing plasmacytoid differentiation. MGG ×100.

pronounced and a Golgi zone is apparent. The TRAP reaction is usually negative. In cases recognized as SMZL, circulating cells may lack villi.

Anaemia and thrombocytopenia may be present. Occasionally they are autoimmune in nature. In one series of patients with SLVL, 7% developed an autoimmune haemolytic anaemia and 2% an autoimmune thrombocytopenia [162]. Other autoantibodies that have been reported include the lupus anticoagulant and anticardiolipin antibodies [163].

About a quarter of patients have a monoclonal immunoglobulin in the serum (most often IgM, less often IgG, and occasionally IgA) while around 15% of patients have a urinary Bence-Jones protein [162,163].

Poor prognostic factors for disease-specific survival are anaemia and a lymphocyte count of more than 16×10^9/l [162].

Immunophenotype

The SLVL/SMZL cell corresponds to a relatively mature B cell with some features suggesting plasmacytoid differentiation (see Table 6.8). SmIg is strongly expressed (IgM and usually IgD) and cytoplasmic immunoglobulin and CD38 [45] are sometimes detected. There is positivity for B-cell-associated antigens such as CD19, CD20, CD22, CD24, CD79a and FMC7. Expression of CD22 is strong. CD79b is positive in about three quarters of patients [131]. Some of the markers characteristic of CLL, specifically CD23 and CD5, are usually negative (in one study 24% and 19%, respectively, were positive) as are CD10, CD103 and HC2 (in one study 23%, 6% and 4%, respectively, were positive) [164,165]. Cases have been reported with CD5 positivity of circulating cells with cells in the spleen being CD5 negative [166]. CD43 is usually weak or negative [46]. CD11c is expressed in about 50% of patients [164,165], CD25 in about a quarter [39,164] and CD72 in the majority [165]. It is important to use a panel of antibodies and to relate immunophenotype to cytology in order to distinguish SLVL/SMZL from CLL, HCL and mantle cell lymphoma.

Histology

Plasmacytoid differentiation is often more prominent in histological sections than in peripheral blood cells. In contrast to CLL, the bone marrow is infiltrated in only about a half of cases. The pattern of infiltration can be either nodular or, in advanced disease, diffuse. The presence of multiple nodules is most characteristic [138]. Nodules in the bone marrow may have a central reactive germinal centre [163]. Intrasinusoidal infiltration is characteristic and can be highlighted by immunohistochemistry. In contrast to CLL, PLL, HCL and hairy cell variant, splenic infiltration is often predominantly in the white pulp and is micronodular – in early cases with selective involvement of the marginal zone

[4,134,167]. There may also be clusters of cells or a scattering of small nodules in the red pulp. On immunohistochemistry the lymphoma cells do not express annexin A1, cyclin D1 or BCL6.

Cytogenetic and molecular genetic features

About 20% of cases have been described as having t(11;14)(q13;q32), the translocation characteristic of mantle cell lymphoma. However, it is now considered, on the basis of splenic histology, that cases with t(11;14) and cyclin D1 expression are more correctly categorized as mantle cell lymphoma [163,168]. A t(11;14)(p11;q32) translocation has been observed in a small number of patients and has been associated with an adverse prognosis [169] and other translocations with a 14q32 breakpoint have occasionally been found. Translocations with 7q22 and 2p11 breakpoints are observed whereas trisomy 12 and 13q14 abnormalities on conventional cytogenetic analysis are uncommon [170]. Del(13)(q14) is more often observed by FISH [171]. Molecular mechanisms of oncogenesis in patients with 7q abnormalities include dysregulation of the *CDK6* gene by proximity to *IGH* in t(7;14)(q21;q32) and by proximity to κ in t(2;7)(p12;q21). Loss of 7q22-36 (particularly 7q31-32) is found in as many as 40% of patients [163]. Abnormalities of 3q have been found in 10–20% of patients [163]. Trisomy 3, better detected by FISH, is found in approaching a fifth of patients with SLVL, this abnormality also being characteristic of other cases of SMZL [172]. A less common recurring cytogenetic abnormality is i(17q) [172]. *RB1* deletion may be detected by FISH analysis [171]. A minority of cases (less than 20%) have *TP53* (17p13) deletion or mutation, which is associated with a worse prognosis [173]. Somatic hypermutation of *IGVH*, a feature of normal splenic marginal zone B cells, has been found in about 50% of patients [163].

Cytogenetic or molecular genetic features associated with a worse prognosis include loss or dysfunction of *TP53*, 7q22-36 loss and unmutated *IVGH* genes [163].

Problems and pitfalls

Diagnosis can be difficult because of the lack of distinctive immunophenotypic and cytogenetic features. The cytoplasmic 'villi' can also be infrequent.

Follicular lymphoma

Follicular lymphoma is a chronic B-lineage lymphoproliferative disorder with a growth pattern in lymph nodes which is, at least in part, follicular. Cells may be predominantly small, mixed small and large, or predominantly large. Although this lymphoma is best defined histologically it has distinctive cytological features and in the great majority of cases characteristic cytogenetic and molecular genetic features are also present. It is thus possible to make a diagnosis without the benefit of histology when there are circulating neoplastic cells. In the WHO classification, follicular lymphoma is divided into grades 1, 2, 3a and 3b, on the basis of the proportion of large cells [174]. Grade 3b follicular lymphoma appears to be a somewhat different disease from grades 1–3a.

Clinical, haematological and cytological features

Follicular lymphoma is a disease of adults, which, unusually for haematological neoplasms, shows a female predominance. Clinical features are localized or generalized lymphadenopathy with hepatomegaly and splenomegaly in those with more advanced disease. Occasionally the disease is diagnosed following an incidental blood film when there are no abnormalities on physical examination.

The circulating lymphoma cells are more pleomorphic than those of CLL. They range in size from cells that are distinctly smaller than those of CLL with small, uniformly condensed nuclei and very scanty cytoplasm, to larger cells with more abundant cytoplasm. Cells may be round or somewhat angular. Nucleoli are usually not visible. A variable but often large proportion of the cells have nuclei with deep, narrow clefts or fissures (Fig. 6.21). Rarely lymphoma cells contain crystalline inclusions [175].

Follicular lymphoma may transform to a large cell lymphoma or, rarely, to Burkitt lymphoma or a lymphoblastic lymphoma/acute lymphoblastic leukaemia [176]. Burkitt transformation is related to the acquisition of a second chromosomal rearrangement, either t(8;14) or t(8;22), leading to *MYC* dysregulation [177].

In addition to the prognostic factors included in the Follicular Lymphoma International Prognostic Index (FLIPI) (see Table 6.6), multivariate analysis shows bone marrow infiltration, male gender and

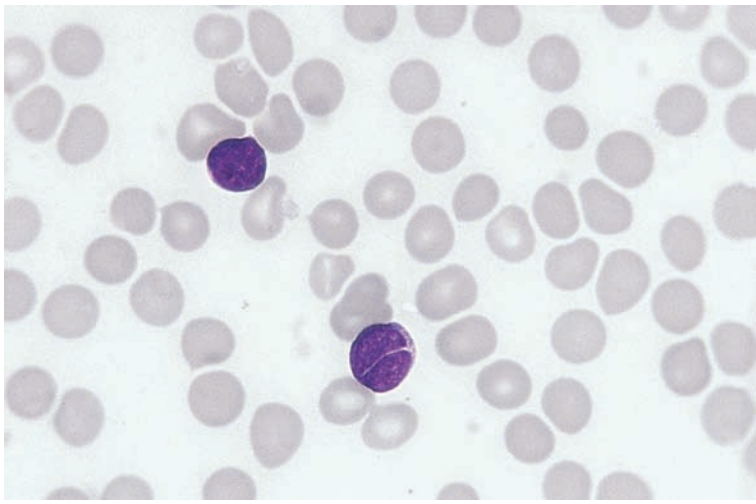

Fig. 6.21 PB film in the leukaemic phase of follicular lymphoma. One cell is very small with scanty cytoplasm; the other is nucleolated and has a deep, narrow cleft. MGG ×100.

a lymphocyte count of less than 1.0×10^9/l to be associated with a worse prognosis [12].

Immunophenotype

The characteristic immunophenotype (Figs 6.22 and 6.23) is positivity for B-cell-associated antigens such as CD19, CD20, CD22 and CD24 and positivity for FMC7. CD79b is usually positive (around 80% of cases) [131] and CD10 is often positive. The detection of CD10 positivity appears to be dependent on the specific antibody-fluorochrome used, in one study being observed to be 100% with one reagent and 0% with another [178]. Use of phycoerythrin (PE) rather than fluorescein isothiocyanate (FITC) is advised, to increase the probability of detecting a weaker reaction [179]. CD5 is not expressed. CD23, CD43 and CD11c are usually negative, although CD43 may be positive in some higher grade follicular lymphomas [46]. On flow cytometry, BCL2 expression is strong whereas reactive B cells show weak expression [180]. SmIg is strongly expressed. IgM is most often expressed but IgG and IgA expression are also quite common; IgD is not expressed [181]; κ expression is more common than λ expression.

Histology

Lymph node histology shows a follicular growth pattern. BCL2 is expressed in the follicles; expression is less common in those with grade 3 histology. However, cases with *BCL6* rather than *BCL2* rearrangement lack *BCL2* expression [182].

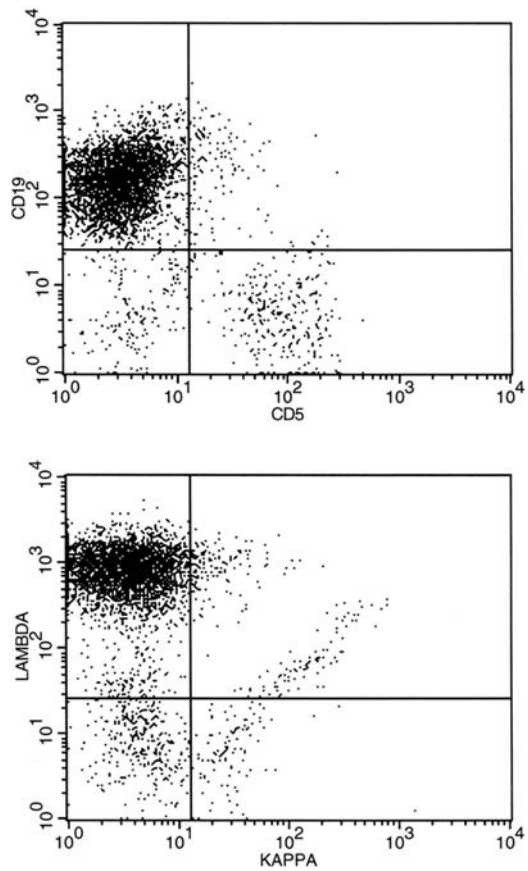

Fig. 6.22 Flow cytometric immunophenotyping in follicular lymphoma showing expression of CD19, strong expression of the. λ light chain and lack of expression of CD5. (By courtesy of Mr Ricardo Morilla, London.)

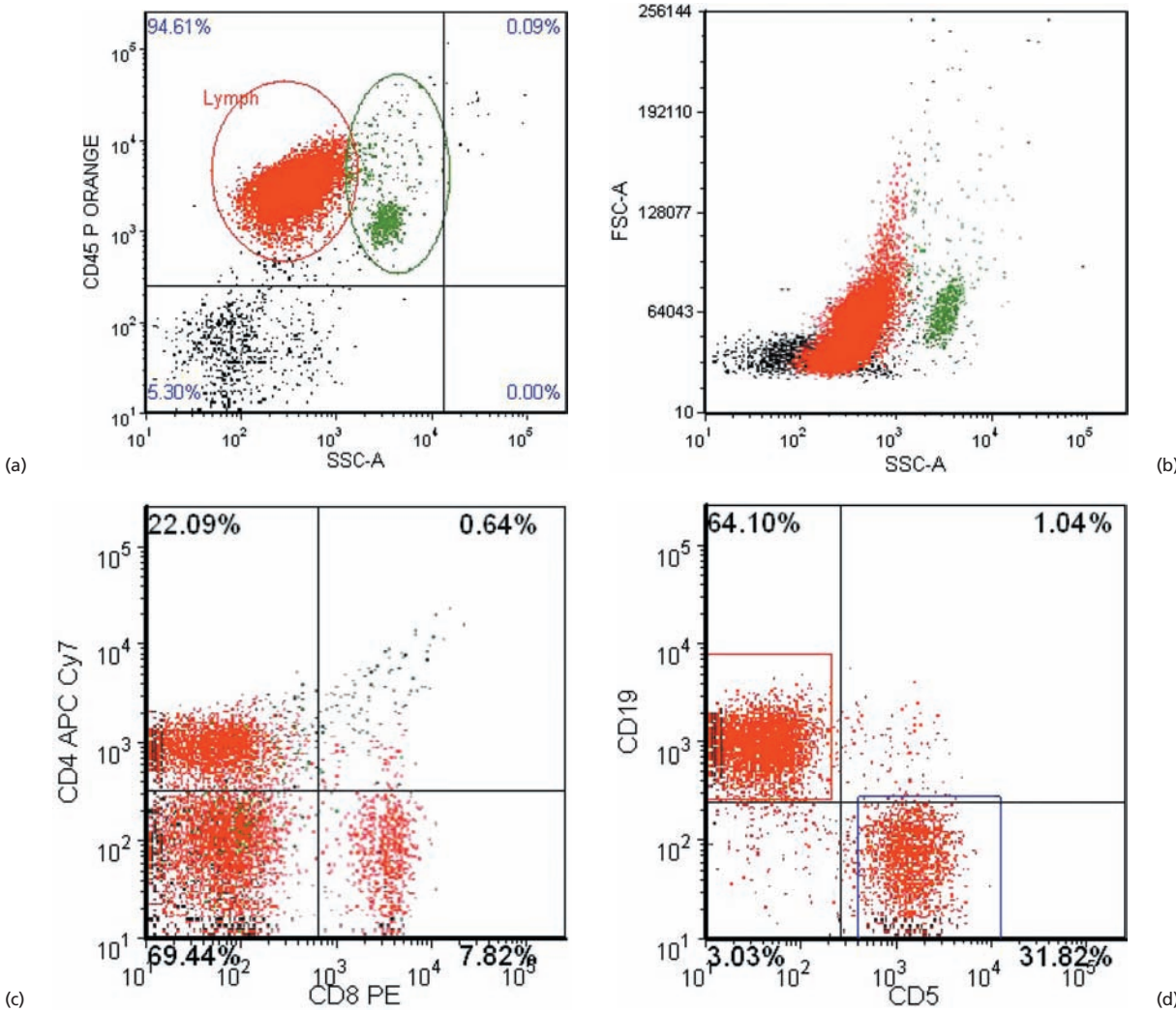

Fig. 6.23 Scatter plots showing flow cytometric immunophenotyping in follicular lymphoma: (a) CD45 against sideways light scatter (SSC) with gating on clusters corresponding to lymphocytes (red) and granulocytes (green); (b) forward light scatter (FSC) against SSC with gating on clusters corresponding to lymphocytes (red) and granulocytes (green); (c) CD4 against CD8 for the gated lymphocyte population only showing CD4+ CD8− T cells, CD4− CD8+ T cells and CD4− CD8− B cells; (d) CD19 against CD5 showing that the CD19+ B cells do not express CD5. (*Continued*)

Bone marrow infiltration is common with paratrabecular infiltration being most characteristic. A nodular growth pattern in the bone marrow is quite uncommon. In advanced disease there is a diffuse infiltrate giving a 'packed marrow' pattern. BCL2 is usually expressed; the combination of CD10 expression and strong BCL2 expression can be used to identify lymphoma cells infiltrating the bone marrow, haematogones having weaker BCL2 expression [180].

Cytogenetic and molecular genetic features

Follicular lymphoma arises from a post-germinal centre B cell showing somatic hypermutation. The most characteristic cytogenetic abnormality is t(14;18)(q32;q21) (Fig. 6.24). Proximity to the *IGH*

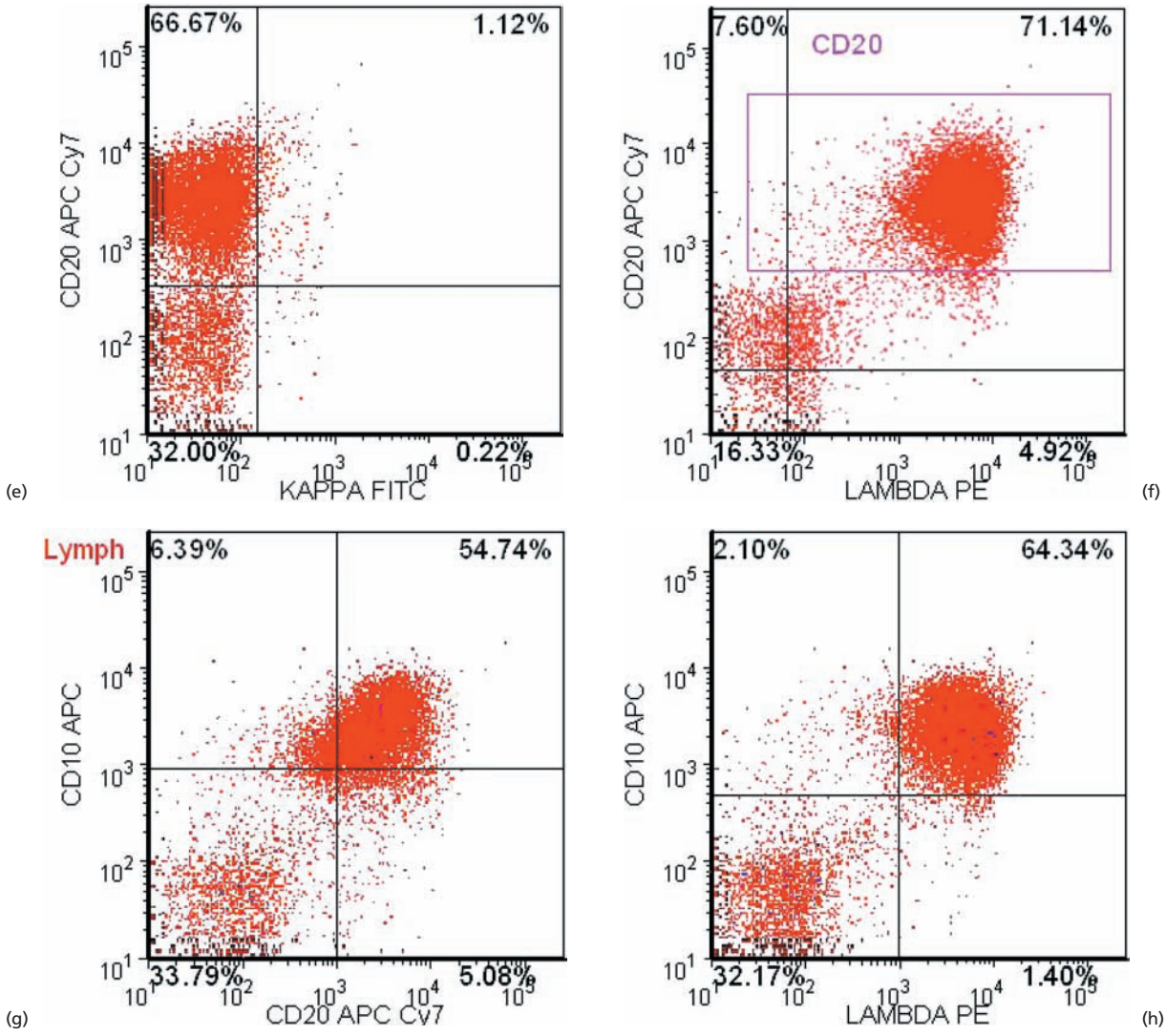

Fig. 6.23 (*Continued*) (e) CD20 against κ showing that the great majority of the B cells do not express κ; (f) CD20 against λ showing that the great majority of the B cells express λ, indicating their clonal nature; (g) CD10 against CD20 showing that the abnormal B cell population express CD10; (h) CD10 against λ confirming that the abnormal λ-expressing clonal B cells express CD10. APC, allophycocyanin; FITC, fluorescein isothiocyanate; PE, phycoerythrin. (By courtesy of Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

locus or associated mutation dysregulates the *BCL2* oncogene at 18q21 rendering the cells resistant to apoptosis. In the variant translocations, t(2;18)(p12;q21) and t(18;22)(q21;q11.2), *BCL2* is dysregulated by proximity to κ and λ genes, respectively. Classical or variant translocations are present in up to 85–95% of follicular lymphomas [182]. There are two clusters of breakpoints in the *BCL2* gene, designated respectively the major breakpoint region (MBR) and the minor cluster region (MCR). Prognosis has been related to the breakpoint, being found to be best with MCR breakpoints, intermediate

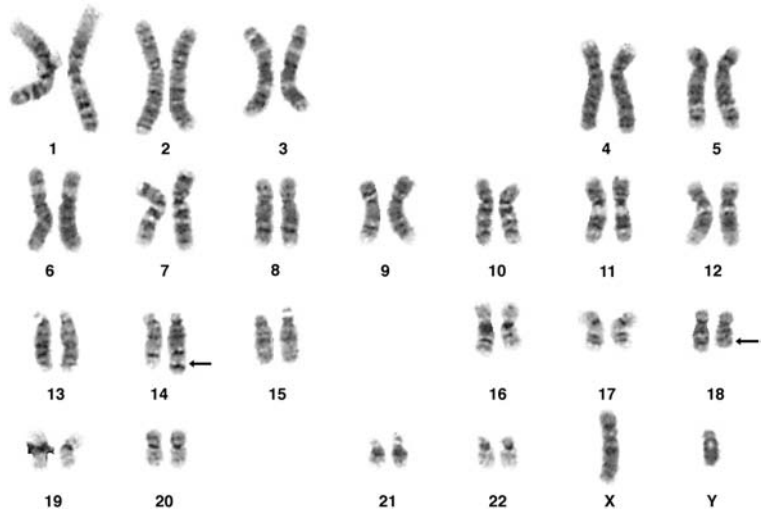

Fig. 6.24 A karyogram showing t(14;18)(q32;q21). (By courtesy of Dr Fiona Ross, Salisbury.)

with MBR breakpoints and worst when neither breakpoint is detected [183]. *BCL2* rearrangement can be detected by both PCR and RT-PCR [184]. When PCR of genomic DNA is used, two separate reactions are needed in order to identify both MBR and MCR breakpoints. t(14;18)(q32;q21) and variant translocations can be detected by single-colour FISH, with a probe that encompasses the breakpoint on chromosome 18. t(14;18)(q32;q21) can be identified more specifically by dual-colour, dual-fusion FISH, using probes that encompass the breakpoints on chromosome 14 and chromosome 18, respectively.

Secondary cytogenetic abnormalities are common and include trisomy of 5, 7, 12, 21 or X, duplication of the der(18), del(6q), del(10q), del(13q) [185], der(1)t(1;17)(p36;q11-21) and der(1)t(1;11)(p36;q13) [186]. Some cases, both with and without t(14;18), show rearrangement of the *BCL6* gene at 3q27 [187]. Rearrangement of *BCL6*, most often resulting from t(3;14)(q27;q32), appears to be an alternative oncogenic mechanism in cases lacking rearrangement of *BCL2* [182]. In comparison with other grades of follicular lymphoma, grade 3b disease is less likely to be associated with *BCL2* rearrangement and more likely to be associated with *BCL6* rearrangement [188]. Trisomy 7 has been related to large cell histology and rearrangements with an 8q24 breakpoint to a blastoid variant [185]. Rearrangement of the *BCL3* gene at 17q22 correlates with transformation to more aggressive disease [189]. Disease progression can also be associated with acquisition of t(1;22)(q22;q11) in which the *FCGR2B* (*FcγRIIB*) gene is brought under the influence of positive regulatory elements of the λ gene [190]. Molecular genetic abnormalities that may appear with disease progression include loss of 6q25-26 [188], loss of *TP53*, which correlates with del(17p), and loss of *CDKN2A* (p16) and *CDKN2B* (p15), which correlates with del(9)(p21). Follicular lymphoma is quite uncommon in children but when it occurs the proportion of patients with *BCL2* rearrangement and *BCL2* expression is higher than in adults [191]; BCL2-negative cases have more limited disease and a much better prognosis.

Problems and pitfalls

The diagnosis of follicular lymphoma is usually straightforward on tissue biopsy except when the growth pattern is diffuse rather than follicular; in these patients supplementary cytogenetic or molecular genetic techniques are needed. In patients with circulating lymphoma cells diagnosis can usually be made reliably from cytology supplemented by immunophenotyping and genetic techniques.

Mantle cell lymphoma

Mantle cell lymphoma is a chronic B-lineage lymphoproliferative disorder with variable cytological and histological features but with a characteristic

cytogenetic and molecular genetic defect. Although histologically low grade, its prognosis is intermediate between that of other low grade B-cell lymphomas (such as follicular lymphoma) and high grade lymphomas (such as diffuse large B-cell lymphoma) with a median survival of only 3–4 years, with the disease generally being incurable.

Clinical, haematological and cytological features

Mantle cell lymphoma, previously referred to as intermediate lymphoma, lymphoma of intermediate differentiation or diffuse centrocytic lymphoma, is principally a disease of middle and old age (median age 60 years) with a marked male predominance [192]. Presentation is usually with advanced stage disease, most often with lymphadenopathy and splenomegaly and often with extranodal disease, including involvement of Waldeyer's ring and the gastrointestinal tract – multiple lymphomatous polyposis [193,194]. There is peripheral blood involvement in about two thirds of patients and this is indicative of a worse prognosis [195]. Central nervous system disease may be more likely in patients with peripheral blood involvement since it was observed in 6 of 58 patients in one series [196]. Cells are usually pleomorphic and most often mainly medium-sized with irregular nuclei [197] (Figs 6.25 and 6.26). Some show inconspicuous nucleoli or pronounced nuclear indentations or clefts. There may be some cells with a diffuse chromatin pattern and in occasional patients these cells predominate [193]. When most cells have a diffuse chromatin pattern, a diagnosis of blastoid variant of mantle cell lymphoma is appropriate; cells in the blastoid variant may be monomorphic, resembling ALL or there may be some more mature cells (Figs 6.27 and 6.28). The blastoid variant usually comprises 10–20% of cases though in some series has been as high as 40% [192,196,198]. In some patients, lymphoma cells have the cytological features of prolymphocytes [136]. Some patients, around 15% of cases, have mainly small cells with only a minority of cells having typical mantle cell features [196]. Platelet satellitism of lymphoma cells has been described [199]. Following splenectomy, the WBC often falls [26]. Patients presenting with typical mantle cell lymphoma features may have a blastoid transformation with disease progression [196]. Rarely transformation is to Burkitt lymphoma [200,201].

Many adverse prognostic indicators are known [192]. These include more advanced age, worse performance status, B symptoms, high WBC, higher lactate dehydrogenase (LDH), blastoid and pleomorphic cytological variants, higher proliferation fraction or mitotic index and adverse karyotype.

Immunophenotype

The characteristic immunophenotype is expression of B-cell-associated antigens such as CD19, CD20, CD22, CD24 and CD79a, expression of CD5, CD79b, FMC7 and BCL2, and lack of expression of

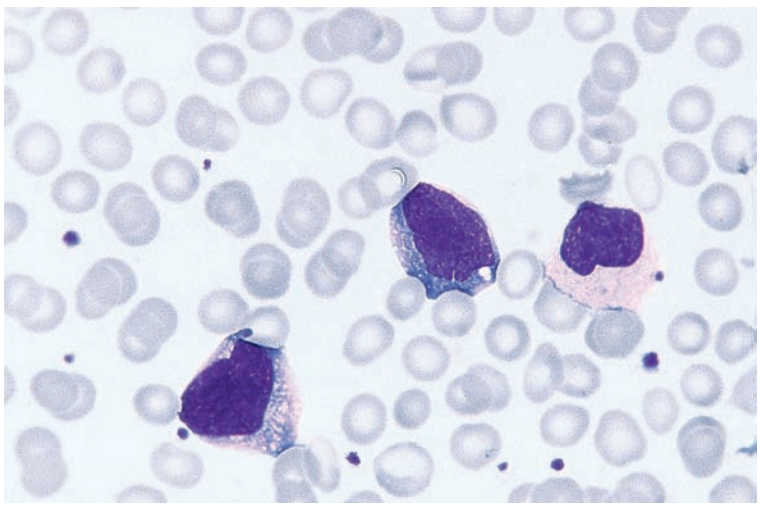

Fig. 6.25 PB film in mantle cell lymphoma. The cells are markedly pleomorphic. MGG ×100.

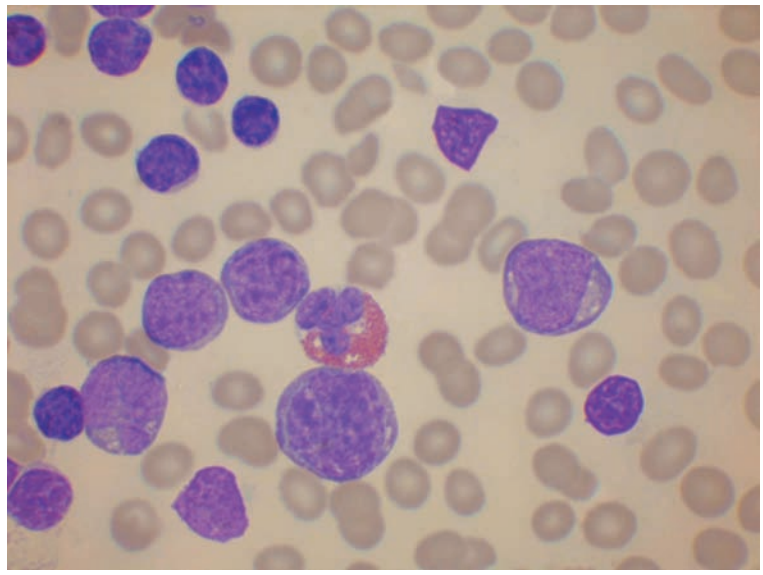

Fig. 6.26 PB film from a patient with mantle cell lymphoma showing a range of cells from small lymphocytes resembling those of CLL to larger cells with irregular nuclei and ill-defined nucleoli; there are some smear cells. MGG ×100.

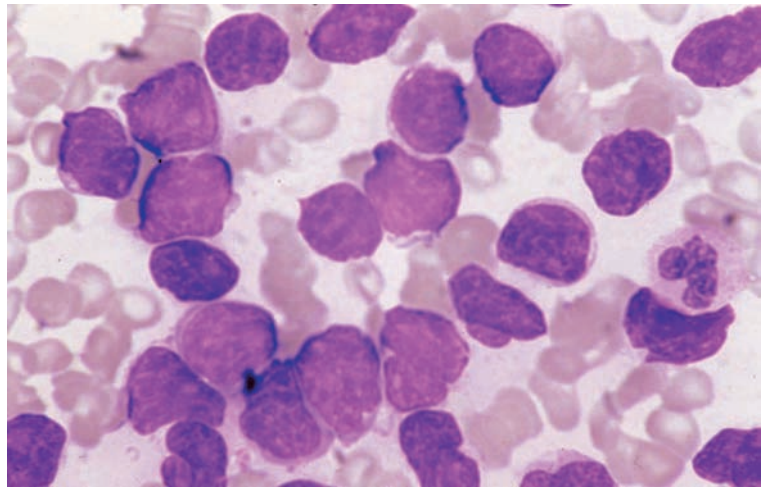

Fig. 6.27 PB film in blastoid variant of mantle cell lymphoma showing medium-sized cells with a high nucleocytoplasmic ratio; some have a diffuse chromatin pattern and others show some degree of chromatin condensation. Some cells have distinct small or medium-sized nucleoli. MGG ×100.

CD10, CD11c, CD103 and BCL6 (see Fig. 2.4). Expression of CD20 is strong. There is failure to express CD5 in 10–15% of patients. CD23 is expressed in up to a half of patients but expression is weak [202,203] and it may therefore be more readily detected by flow cytometry than by immunohistochemistry. CD25 is usually not expressed. CD38 is expressed on more than 20% of cells in the majority of patients [204], and correlates with a worse prognosis [205]. CD43 is variously stated to be weak or negative [204] or usually strong [46,206]. CD45 expression is strong. There may be aberrant expression of myeloid and T-lineage antigens such as CD2, CD7, CD13 and CD33 [207]. SmIg is moderately strongly expressed. It is usually IgM with or without IgD. λ is more commonly expressed than κ [181]. The cells of mantle cell lymphoma usually express nuclear cyclin D1 (see below), which can be detected immunocytochemically and, if cells are 'permeabilized', by flow cytometry [208,209]). However, the detection of nuclear cyclin D1 by flow cytometry may be neither very sensitive (in one

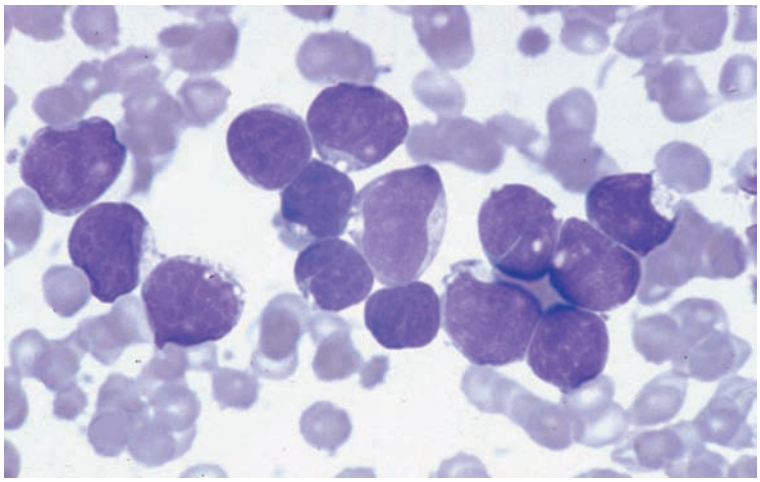

Fig. 6.28 PB film from a patient with blastoid variant of mantle cell lymphoma showing pleomorphic cells ranging from mature lymphocytes to blast-like cells. Some cells have cleft nuclei and some have cytoplasmic vacuoles. MGG ×100.

study positive in only two thirds of patients) nor totally specific (in the same study also positive in a fifth of patients with CLL) [209].

Histology

Bone marrow infiltration is common, being observed in up to 80% of patients [195]. The pattern of infiltration is usually interstitial or focal with either nodules or irregularly shaped infiltrates. Paratrabecular infiltration is uncommon. Lymphoma cells in trephine biopsy sections may have regular round nuclei or irregular and cleaved nuclei. Chromatin is relatively dense and nucleoli are inconspicuous. Some cases have a high mitotic rate or cells with more blastic morphology, both these features being indicative of a worse prognosis. In the blastoid variant there may be a 'starry sky' appearance [192]. On immunohistochemistry, CD5 expression is not always detected, even when it is detected by flow cytometry. Immunohistochemical demonstration of nuclear cyclin D1 expression is diagnostically useful [210], being detectable in almost all cases [211]. A higher proliferative fraction (Ki-67 expression) and expression of p53 are indicative of a worse prognosis [211]; both are more often observed in the blastoid variant [198].

Lymph node histology may show a mantle zone, nodular or diffuse pattern of infiltration. Mantle zone infiltration, surrounding a reactive germinal centre, may be the earliest pattern with later invasion of the reactive germinal centre (giving a nodular pattern) and subsequent obliteration of the lymph node. Splenic infiltration is mainly in the white pulp.

Cytogenetic and molecular genetic features

The characteristic cytogenetic abnormality is t(11;14)(q13;q32) (Fig. 6.29) leading to dysregulation of the *CCND1* gene (encoding cyclin D1), which is brought into proximity to the *IGH* locus with resultant overexpression of cyclin D1. This rearrangement is not specific for mantle cell lymphoma, being reported also in multiple myeloma; however, in the right cytological/histological context it is regarded as diagnostic of mantle cell lymphoma. This translocation is detected in many cases of mantle cell lymphoma by conventional cytogenetic analysis but in almost all if FISH techniques are employed [212]. Many FISH techniques are applicable. FISH can be performed with a whole chromosome paint for chromosome 11, with a single probe spanning the breakpoints of *CCND1* or with two probes, one centred on the *CCND1* gene and the other being a chromosome 11 centromeric probe [213]. Alternatively, specific probes for *CCND1* and *IGH* can be used, with co-localization of signals being seen. Probes that encompass each gene are available, permitting dual-colour, dual-fusion FISH. Genomic PCR usually detects *CCND1* rearrangement in slightly more than 50% of patients, specifically those with breakpoints in the major translocation cluster (MTC) on chromosome 11.

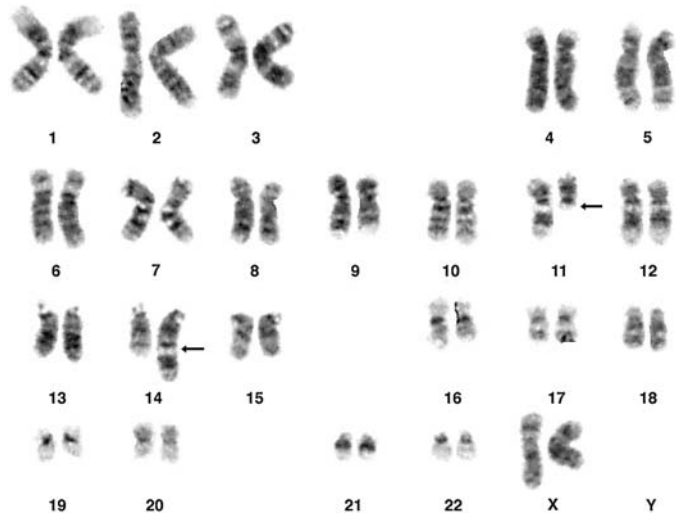

Fig. 6.29 A karyogram showing t(11;14)(q13;q32). (By courtesy of Dr Fiona Ross.)

RT-PCR is also applicable [184] but false-negative results occur. A minority of cases of mantle cell lymphoma show a variant translocation, t(11;22)(q13;q11), which also leads to dysregulation of cyclin D1, in this case as a result of juxtaposition to the λ light chain locus. Mantle cell lymphoma can also arise as the result of overexpression of cyclin D2 or cyclin D3. For example, patients have been described in whom cyclin D2 overexpression resulted from t(2;12)(p12;p13), bringing *CCND2* (encoding cyclin D2) under the influence of the κ locus [214], or t(12;14)(p13;q32), bringing *CCND2* under the influence of the *IGH* locus [215].

It is unusual for t(11;14) to be present as the sole abnormality [216]. In one study 85% of patients had extra chromosomal abnormalities [198]. Common secondary cytogenetic abnormalities include del(1p), del(6q)(22-23), add(3q)(26-29), +3, add(8q), −9, del(9p), del(10q), del(11)(q23), add(12q), +12, del(13)(q14), del(13)(q31-34), del(17)(p13), −13 and −18 [211,216–220]. Although trisomy 12 may be observed in mantle cell lymphoma, as well as in CLL, trisomy 12 as a sole abnormality is not a feature of mantle cell lymphoma. Tetraploidy was reported to be common in the blastoid variant in two series of patients [198,221] but not in a third series [216]. Burkitt lymphoma-related translocations, both t(8;14)(q24;q32) and t(2;8)(p12;q24), have occurred in blastoid transformation [222] and in transformation to Burkitt lymphoma [200]. Certain cytogenetic abnormalities correlate with significant numbers of circulating lymphoma cells, specifically abnormalities of 17, 21 and 22 and rearrangements with 8q24, 9p22-24 and 16q24 breakpoints [216]. Trisomy 12 [223], add(Xq) [224], del(17p) [224] or specifically del(17)(p13) [198], del(9)(p21) [198], add(3q) [192] and complex karyotypes [223] have been associated with a worse prognosis.

Cells of mantle cell lymphoma usually show unmutated *IGVH* (pre-germinal centre origin) genes but in a significant minority there is *IGVH* somatic hypermutation [194]. The presence of hypermutated genes has been found to correlate with predominantly non-nodal disease and to include a subgroup of patients with indolent disease, whose cells may express CD38 [225]. In addition to dysregulation of the *BCL1* gene, there is often amplification of 3q28q29 suggesting that another oncogene at that site may be relevant [224]. Amplification of part of 6p is also common [224]. Chromosomal regions that often show deletions include 1p13p32, 5p13p15.3, 6q14q27, 8p,11q13q23 (particularly 11q22.1-23.3) and, most frequently, 13q (13q14 and 13q34) [224,226]. Del(8p) and del(13)(q34) correlate with a worse prognosis [226]. Inactivation of the *ATM* gene at 11q23, by point mutation or deletion, has been observed in some patients [194,218]; in patients with loss of one allele of *ATM* as a result of del(11)(q23) the other

allele is often mutated [110]. A loss of 8p21-p23 was associated, in one study, with mantle cell lymphoma with leukaemic manifestations, suggesting that there may be a tumour suppressor gene at this locus [219] but, in a second study, association with leukaemic manifestations was not observed [226]. Patients with the blastoid variant often have mutation of *TP53*, *CDKN2A* (encoding $p16^{INK4A}$) and *CDKN2C* (encoding $p18^{INK4C}$) [224]. Mutation of *CDKN1A* (encoding p21) is also associated with worse prognosis [227]. Expression of p53 correlates with *TP53* mutation and is indicative of a worse prognosis [205].

Problems and pitfalls

Diagnosis from cytological features is difficult. The blastoid variant can be confused with ALL and cases with uniform small cells with CLL. Cases with more pleomorphism can resemble the mixed cell type of CLL. In the absence of histology, cytology must be integrated with immunophenotypic and genetic information to reach a diagnosis. Although a typical immunophenotype can be defined, a significant minority of patients with mantle cell lymphoma have a surface membrane immunophenotype very similar to that of CLL. Demonstration of t(11;14) or of nuclear cyclin D1 expression is useful for the identification of such cases [204,228].

Burkitt lymphoma

'Acute leukaemia with Burkitt lymphoma cells' was first described in 1972 [229], although the occurrence of bone marrow infiltration and a terminal leukaemic phase of endemic African Burkitt lymphoma had been recognized earlier than this. In the FAB classification the cytological features of Burkitt lymphoma were recognized as 'L3 ALL' but since the neoplastic cells are now known to be mature B cells, not precursor cells, classification as ALL is no longer appropriate. Burkitt lymphoma is a highly aggressive lymphoma that is often curable with specific treatment schedules. Its rapid recognition is therefore important and observation of L3 cytological features in a blood film or a bone marrow aspirate should lead to urgent immunophenotyping and genetic analysis. More often the diagnosis is made on histological examination of a tissue biopsy.

Burkitt lymphoma occurs in an endemic form, a sporadic form and a human immunodeficiency virus (HIV)-related form. Endemic Burkitt lymphoma occurs in equatorial Africa and in Papua and New Guinea. Epidemiologically it is very strongly linked to holoendemic malaria and EBV is found in lymphoma cells. Sporadic Burkitt lymphoma is EBV associated in 10–30% of cases and in HIV-related disease there is EBV association in 30–40% of cases. EBV-positive Burkitt lymphoma arises from a post-germinal centre memory B cell whereas EBV-negative disease arises from a germinal centre B cell [230].

In addition to *de novo* cases, Burkitt lymphoma can also occur as a transformation of a lower grade leukaemia or lymphoma, e.g. CLL, follicular lymphoma or mantle cell lymphoma.

Clinical, haematological and cytological features

Clinical presentation varies between endemic cases (jaw tumours and cervical lymphadenopathy), sporadic cases (often intestinal disease) and HIV-related cases (generally widespread disease with lymphadenopathy). Bone marrow infiltration and a leukaemic phase are uncommon in endemic and sporadic cases but are much more often seen in HIV-associated cases. The neoplastic cells are medium sized with a high nucleocytoplasmic ratio; cytoplasm is strongly basophilic and moderately vacuolated. Bone marrow aspirates show many mitotic figures. Adverse prognostic features include bone marrow and central nervous system disease, a high LDH and a large unresected tumour mass [231].

Immunophenotype

The lymphoma cells are mature B cells, expressing IgM, B-cell-associated antigens (e.g. CD20, CD22, CD79a and CD79b) and CD10. CD34 and TdT are not expressed.

Histology

On histological sections, cytoplasmic basophilia is detectable (optimally on a Giemsa stain) but vacuolation is much less apparent than in cytological preparations. There are frequent mitotic figures and also many apoptotic cells. Numerous macrophages containing apoptotic debris create a 'starry sky' appearance. Immunohistochemical demonstration of expression of BCL6 but not BCL2 supports the diagnosis of Burkitt lymphoma; BCL2 is weakly

positive in only 20% of patients [231]. Demonstration of expression of the proliferation marker, Ki-67, is important in confirming the diagnosis; expression approaches 100%.

Cytogenetic and molecular genetic features

The majority of patients have t(8;14)(q24;q32) with juxtaposition of *MYC* to the *IGH* locus (see Fig. 2.10). In a minority of patients there is t(2;8)(p12;q24) or t(8;22)(q24;q11.2) with juxtaposition of *MYC* to the κ and λ loci respectively. These rearrangements can be demonstrated by FISH (Fig. 6.30). The gene expression profile is distinctive.

Problems and pitalls

L3 cytological features do not always equate with Burkitt lymphoma. Immunophenotyping and genetic analysis are essential to confirm the diagnosis.

Diffuse large B-cell lymphoma and other lymphomas of large B cells

Diffuse large B-cell lymphoma (DLBCL), despite being a neoplasm of immunophenotypically mature cells, is clinically a moderately aggressive tumour. Presentation is usually with lymphadenopathy or extranodal disease but in occasional cases there is peripheral blood and bone marrow involvement. The 2008 WHO classification recognizes DLBCL, not otherwise specified [232], and a number of less common specific subtypes, which are even less likely to involve the blood. Some cases represent either transformation of a lower grade lymphoma or a clonally unrelated neoplasm that occurs in the context of immunosuppression related to a lower grade leukaemia/lymphoma.

Clinical, haematological and cytological features

Most patients with DLBCL present with lymphadenopathy, sometimes with associated hepatosplenomegaly. A small minority present in leukaemic phase or develop lymphoma cell leukaemia with disease progression [233]. In these patients impairment of bone marrow function may cause anaemia and cytopenia. Lymphoma cells may be present in relatively small numbers or may be very numerous. Pleomorphism is common. The cytological features are very variable from case to case. The cell outline may be either regular or irregular. Cytoplasm is often plentiful and either weakly or strongly basophilic. Nuclei may be round, irregular, lobulated or cleft (Fig. 6.31). Chromatin may be mainly diffuse or show condensation. Nucleoli are common and may be conspicuous. A rare observation in intravascular large B-cell lymphoma is of clumps of tumour cells revealed when a film is made from the tip of the needle used for phlebotomy [234] (Figs 6.32 and 6.33). Adverse prognosis is associated with higher patient age, poor performance status, more advanced stage or more bulky disease, higher LDH, bone marrow involvement and a germinal centre rather than activated B-cell-like gene expression profile (see below) or equivalent immunophenotypic profile. A reduced lymphocyte count

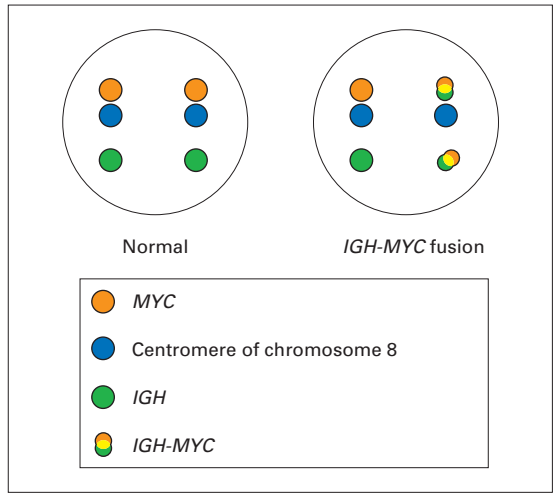

Fig. 6.30 Diagrammatic representation of tricolour, dual-fusion FISH for the detection of *IGH-MYC* juxtaposition, using an orange *MYC* probe, a green *IGH* probe and a blue probe for the centromere of chromosome 8. The normal cell has two orange *MYC* signals, two blue centromeric signals and two green *IGH* signals. The cell with *IGH-MYC* juxtaposition as a result of t(8;14) has one normal orange *MYC* signal, a normal blue centromeric signal, a normal green *IGH* signal, two fusion *IGH-MYC* signals and a second blue centromeric signal adjacent to one of the fusion signals. Rearrangements In the *MYC* region can also be detected using a dual-colour, break-apart FISH technique In which *MYC* is identified with a dual-colour, orange–yellow–green probe; when rearrangement has occurred, two distinct orange and green signals are seen. This second strategy will detect rearrangements in the *MYC* region occurring with t(8;22)(q24;q11.2) and t(2;8)(p12;q24) as well as with the more common t(8;14)(q24;q32).

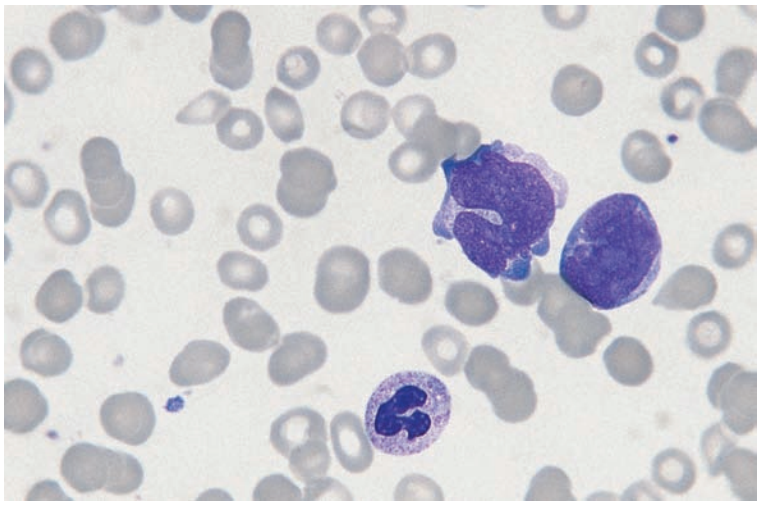

Fig. 6.31 PB film in diffuse large B-cell lymphoma of centroblastic type. MGG ×100.

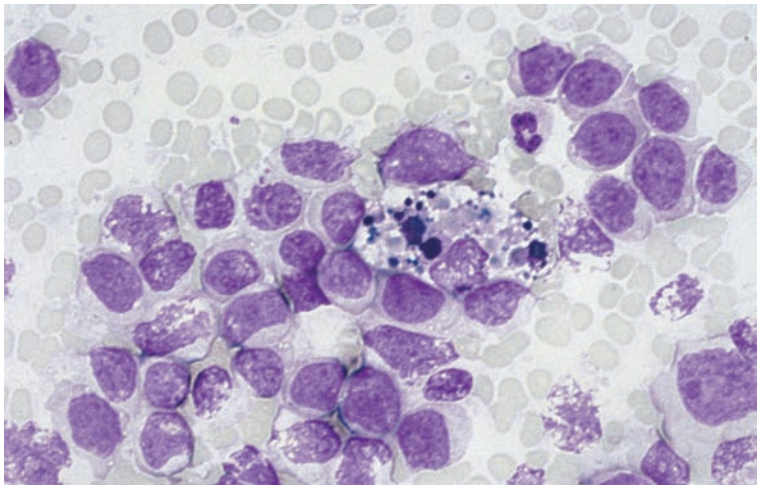

Fig. 6.32 A clump of lymphoma cells and a macrophage in a blood film of a patient with intravascular large B-cell lymphoma. The blood film was prepared from the tip of the needle following phlebotomy. MGG ×100. (By courtesy of Dr Ralph Cobcroft, Brisbane.)

(<0.8×10^9/l) has also been found to be prognostically adverse [235].

Immunophenotype

Flow cytometry shows high forward angle light scatter [145]. The immunophenotype is that of a mature B cell but the expression of specific immunophenotypic markers varies from case to case, reflecting the heterogeneity of this condition. There is variable expression of B-cell-associated antigens and of CD5 and CD10, while CD34 and TdT are not expressed. CD10 expression may correlate with follicular centre origin [178]. CD71 is usually expressed [145]. There may be a failure to express SmIg [145].

Histology

The bone marrow is consistently involved in patients with circulating lymphoma cells but the pattern of infiltration varies. Lymph nodes usually show diffuse infiltration but occasional cases have a follicular pattern. There is expression of CD20 and CD79a and, in some patients, CD5, CD10, CD30, BCL2, BCL6, IRF4/MUM1 and p53 [232].

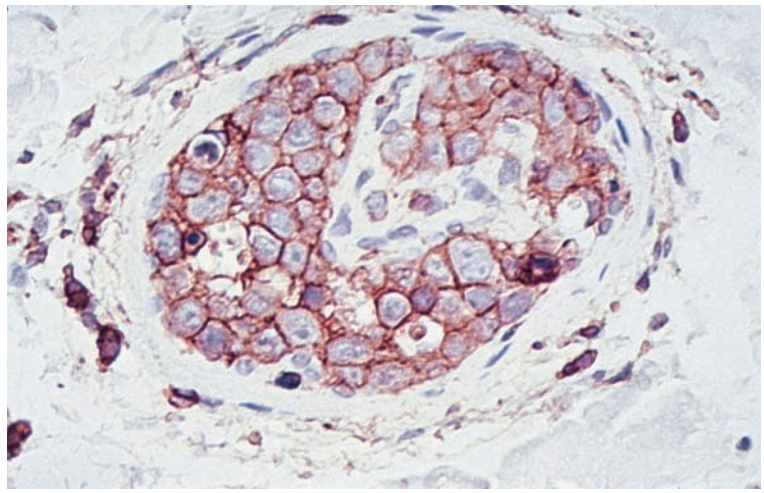

Fig. 6.33 A skin biopsy from a patient with intravascular large B-cell lymphoma showing cohesive masses of lymphoma cells within a capillary (same patient as Fig. 6.32). The cells were CD45 and CD20 positive and S100 negative. Immunohistochemistry with an anti-CD20 monoclonal antibody. (By courtesy of Dr Ralph Cobcroft and the *British Journal of Haematology*.)

Cytogenetic and molecular genetic features

The commonest cytogenetic abnormalities are those characteristic of follicular lymphoma. The second most common group of abnormalities are those with rearrangement of the *BCL6* gene at 3q27, including t(3;14)(q27;q32). Other cases show miscellaneous cytogenetic abnormalities including some with 14q32 (*IGH*) or 8q24 (*MYC*) involvement. Dual-colour FISH with probes for *BCL6* and *IGH* can be used to detect t(3;14)(q27;q32) [236]. Dual-colour, break-apart FISH permits the detection of rearrangement of *BCL6*, *MYC* and the *IGH* locus.

Molecular genetic abnormalities include rearrangement of *IGH*, *BCL2*, *BCL6* and *MYC* and *TP53* mutations. Gene expression profiling by microarray analysis shows that DLBCL can be divided into two major groups by different patterns of gene expression; in one group there is a germinal centre pattern of expression whereas in the other group the pattern of expression resembles that of an activated B cell. The prognosis is better in the former group when patients are treated with combination chemotherapy regimes of the CHOP type (cyclophosphamide, doxorubicin, vincristine, prednisolone) with or without rituximab.

Problems and pitalls

Immunophenotyping is important in recognizing a leukaemic presentation of large cell lymphoma since some cases have cells with cytological similarities to monoblasts. In the absence of immunophenotyping, a non-specific esterase stain is useful for making this distinction. In addition, DLBCL is cytologically indistinguishable from T-lineage large cell lymphoma and can sometimes be confused with cases of plasma cell leukaemia with cytologically very undifferentiated cells.

Lymphoplasmacytoid lymphoma

Lymphoplasmacytoid lymphomas arise from a post-germinal centre, somatically mutated B cell. The circulating neoplastic cells are usually small mature lymphocytes with some plasmacytoid features such as cytoplasmic basophilia, a small Golgi zone or an eccentric nucleus (Fig. 6.34). As defined in the WHO classification, this lymphoma shows some differentiation to mature plasma cells. Occasionally lymphoma cells contain cytoplasmic crystals or spherical inclusions (Russell bodies). In addition, there may be peripheral blood features resulting from the presence of a paraprotein (often but not always IgM) such as increased rouleaux formation, the presence of red cell agglutinates or, less often, a precipitated cryoglobulin between cells or within neutrophils or monocytes.

Clinical features can include splenomegaly, lymphadenopathy and features of hyperviscosity. The bone marrow may show increased mast cells, in addition to a lymphoid infiltrate. Some patients are carriers of hepatitis C.

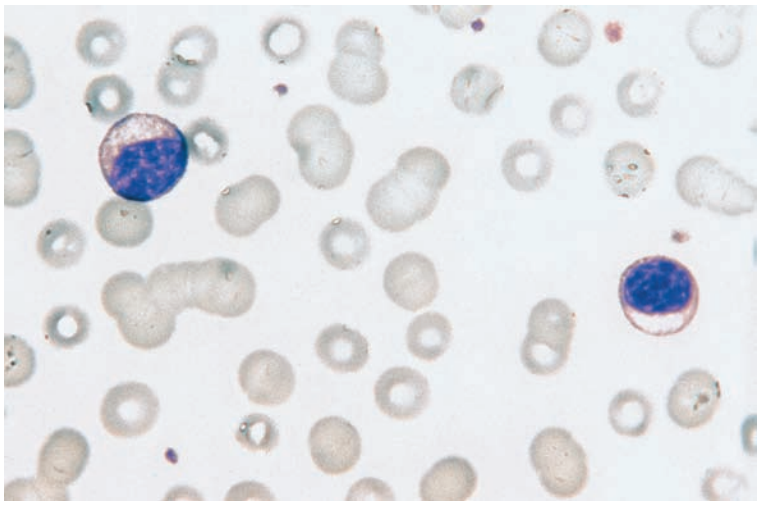

Fig. 6.34 PB film in Waldenström macroglobulinaemia; this term describes a lymphoplasmacytoid lymphoma with production of large amount of monoclonal IgM. The blood film shows two plasmacytoid lymphocytes together with rouleaux and abnormal staining characteristics consequent on the high level of IgM. MGG ×100.

The immunophenotype is that of a late B cell with expression of cytoplasmic immunoglobulin in at least some cells. SmIg is expressed more strongly than in CLL cells. CD11c, CD19, CD20, CD22, CD79a and FMC7 are expressed, with CD20 being more strongly expressed than in CLL [237]. CD5, CD10 and CD23 are not usually expressed (about 5% of cases are CD5 positive). CD38 may be expressed but expression is weaker than in plasma cells [45]. A minority of cells may show strong expression of CD38 and CD138, indicating plasmacytic differentiation. There is often expression of CD11c and CD25 but not of CD103 [179].

The t(9;14)(p13;q32) translocation in which the transcription factor gene, *PAX5*, is dysregulated by proximity to *IGH* has been reported as characteristic [238] but has been found to be actually quite uncommon [239].

It should be noted that plasmacytic differentiation can occur in other types of NHL, e.g. in SMZL.

Heavy chain diseases

The heavy chain diseases are rare lymphoproliferative disorders characterized by synthesis of a defective immunoglobulin heavy chain [240]. In the case of γ and μ heavy chain diseases, the peripheral blood and bone marrow may be involved.

Clinically and histologically, γ heavy chain disease resembles lymphoplasmacytic lymphoma. Associated autoimmune disease is common. The peripheral blood features may resemble CLL or there may be plasmacytoid lymphocytes. The bone marrow may show increased plasma cells or plasmacytoid lymphocytes.

Haematologically, μ heavy chain disease resembles CLL but clinically there is usually hepatosplenomegaly without peripheral lymphadenopathy [240]. The bone marrow aspirate shows not only small lymphocytes but also vacuolated plasma cells [240].

Other non-Hodgkin lymphomas in leukaemic phase

Various other lymphomas may have a leukaemic phase but this is quite uncommon [1]. This has occasionally been reported in marginal zone lymphoma including nodal marginal zone lymphoma (monocytoid B-cell lymphoma) [241] and MALT (mucosa-associated lymphoid tissue) lymphoma [242]. Cytological features are variable and not distinctive. The immunophenotype is usually CD5 negative (only about 5% are positive) and CD10 negative [179]. There is often expression of CD11c (weaker than in HCL) and sometimes of CD103 [179]. There may be some plasmacytoid differentiation leading to expression of CD38 and CD138 in some cells [179].

A leukaemic phase may develop in patients who initially present with nodal small lymphocytic lymphoma. When this occurs the cells have the same

cytological and immunophenotypic features as CLL cells [181], this disease being the tissue equivalent of CLL and classified with it.

Plasma cell leukaemia

Plasma cell leukaemia may occur *de novo* or as the terminal phase of multiple myeloma. The FAB group [4] suggested that the term plasma cell leukaemia be confined to *de novo* cases but in the WHO classification both *de novo* and secondary cases are included [243]. In *de novo* cases, the patients have an acute illness, sometimes with hepatosplenomegaly and often with hypercalcaemia and renal failure. Other differences, for example in immunophenotype and in the range of cytogenetic abnormalities, supports the view that *de novo* plasma cell leukaemia is a different disease from multiple myeloma [244]. Prognosis is poor both in *de novo* cases and secondary cases. In one series of 18 patients with *de novo* disease the median survival was only 7 months [245]. In another series median survival was 22 months in *de novo* cases in comparison with 1.3 months when there had been preceding multiple myeloma [246].

Criteria previously suggested for a diagnosis of plasma cell leukaemia were the presence of circulating neoplastic plasma cells that both: (i) constituted at least 20% of circulating cells; and (ii) had an absolute count of at least 2×10^9/l. In the WHO classification, either of these criteria is accepted as sufficient for this diagnosis [243].

Clinical, haematological and cytological features

Cytological features vary considerably between cases. Some patients have mainly cells that resemble normal plasma cells with basophilic cytoplasm, a prominent Golgi zone and an eccentric nucleus (Fig. 6.35). Others have many lymphoplasmacytoid lymphocytes and only a minority of characteristic plasma cells. Yet others have more primitive cells with a higher nucleocytoplasmic ratio, a diffuse chromatin pattern, a prominent nucleolus and a less prominent Golgi zone (Fig. 6.36). In the latter group it can be difficult to recognize cells as plasma cells by light microscopy alone; in some cases there are cytological similarities with prolymphocytic leukaemia or the immunoblastic subtype of DLBCL.

Immunophenotype

In addition to the markers shown in Table 6.8, positive reactions are found with McAb that show some selectivity for plasma cells such as PCA-1, BU11 [4], CD38 and CD138 [247]. Strong expression of CD38 with weak expression of CD45 is typical of plasma cells, with negative or weak CD45 expression being more common in neoplastic plasma cells. Of the pan-B markers, CD19, CD20 and CD22 are usually negative whereas CD79a is sometimes positive.

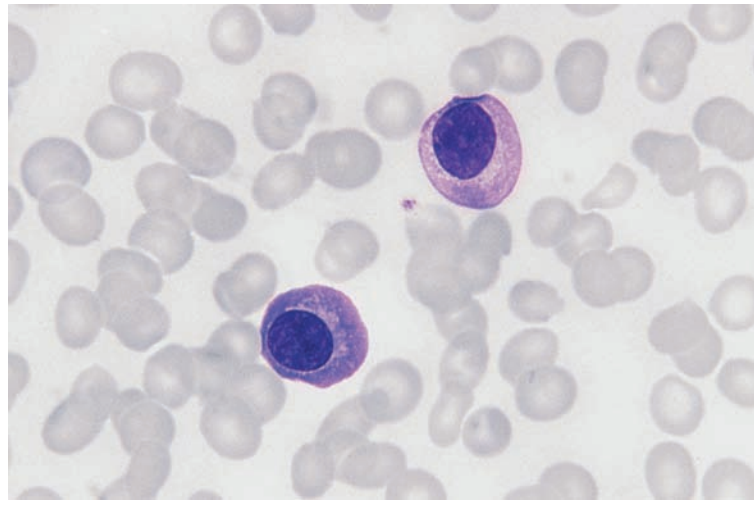

Fig. 6.35 PB film in plasma cell leukaemia. The malignant cells are identified as plasma cells by their eccentric nuclei and pale paranuclear area that represents the Golgi zone. MGG ×100.

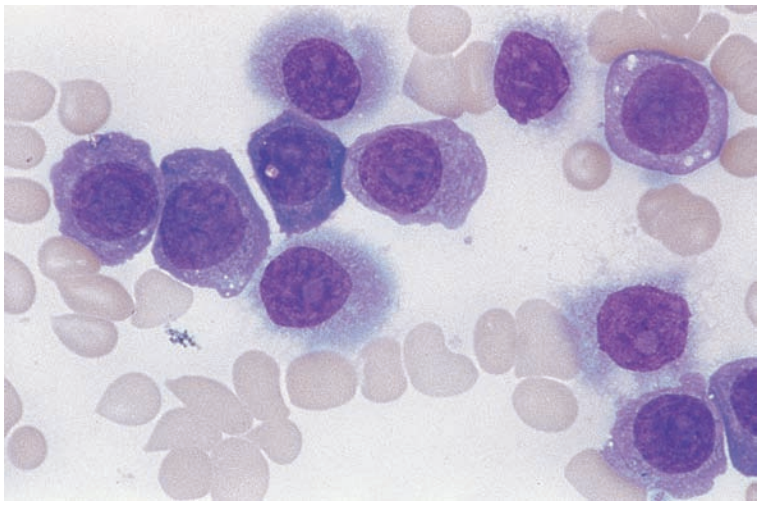

Fig. 6.36 PB film in plasma cell leukaemia with cells showing plasmablastic morphology. MGG ×100.

HLA-DR may be expressed. CD56, which is often aberrantly expressed in multiple myeloma, is not usually expressed in plasma cell leukaemia [243]. There may be aberrant expression of CD13 [244] and CD28 [179] and a failure to express CD27 [179].

The immunophenotype in *de novo* plasma cell leukaemia has been found to differ somewhat from that of multiple myeloma [244]. CD20 is more often expressed whereas CD9, CD56 and HLA-DR are less often expressed [244,246]. Aberrant expression of CD117 (about 20% of cases) may be a feature of cases following multiple myeloma but not of *de novo* plasma cell leukaemia [179,244,246].

Histology

The appearance of histological sections of trephine biopsies or other tissues varies, depending on the degree of maturation of cells. Some cases have infiltrating cells with obvious plasma cell differentiation. In other cases the histological features are similar to those of DLBCL of immunoblastic subtype and demonstration of monotypic cytoplasmic immunoglobulin and CD138 expression is then useful in confirming the diagnosis. Epithelial membrane antigen (EMA) may be expressed, as may CD43 and CD30.

Cytogenetic and molecular genetic features

Plasma cell leukaemia may show cytogenetic abnormalities similar to those of multiple myeloma including t(11;14)(q13;q32) and other rearrangements with a 14q32 breakpoint and, in addition, rearrangements of chromosomes 1 and 11. However there are some differences in the frequency of various abnormalities in multiple myeloma and *de novo* plasma cell leukaemia [244]. Hyperdiploidy is common in multiple myeloma but not in plasma cell leukaemia [244]. Trisomy 1 and trisomy 18 are common in both but trisomies 6, 9 and 15 are more common in multiple myeloma, while plasma cell leukaemia is more likely to show monosomy 1, monosomy 13 and monosomy X [244].

Leukaemias of mature T and NK cells

Leukaemias of mature T and NK cells are quite uncommon, constituting only a small proportion of chronic lymphoid leukaemias. Mature T-lineage leukaemias express one or more T-lymphocyte markers – commonly CD2, CD3, CD5 and either CD4 or CD8 – and show rearrangement at one or more of the *TCR* loci (Tables 6.16 and 6.17). TdT, CD1 and CD34 are not expressed. Leukaemias of NK-cell lineage express surface antigens characteristic of NK cells; they may share some antigens with T-cell leukaemias but do not express CD3 and do not show rearrangement of *TCR* genes. In neither the T nor the NK lineage is there a readily available marker of monoclonality equivalent to the light chain restriction of SmIg of the B lymphocyte. The

Table 6.16 Some monoclonal antibodies used in the characterization of chronic lymphoid leukaemias of T and natural killer (NK) lineages.

Cluster designation	Specificity within haemopoietic and lymphoid lineages
CD2	Receptor for sheep red blood cell; detects E rosette-forming cells (ERFC); positive in all except the earliest of T-lineage cells and on NK cells
CD3	Part of the T-cell receptor complex; expressed on thymocytes and T cells; expressed in the cytoplasm before it is expressed on the cell surface
CD5	Expressed on thymocytes and T cells (see Table 6.7)
CD7	Expressed on pluripotent stem cells, thymocytes and T cells; expressed in cells of some cases of acute myeloid leukaemia
CD4	Common and late thymocytes, subset (about two thirds) of mature T cells (among which are many cells that are functionally helper/inducer) that recognize antigens in a class II context; expressed on monocytes and macrophages
CD8	Common and late thymocytes, subset (about one third) of mature T cells (among which are many cells that are functionally cytotoxic/suppressor) that recognize antigens in a class I context
CD11b	C3bi complement receptor: expressed on monocytes, granulocytes, NK cells and hairy cells
CD16	Component of low-affinity Fc receptor, FcRIII: expressed on NK cells, neutrophils, macrophages
CD56	NK cells, activated lymphocytes, cells of some cases of acute myeloid leukaemia, myeloma cells, some cases of small cell carcinoma of lung
CD57	NK cells, subsets of T cells, B cells and monocytes
CD25, CD38, HLA-DR	See Table 6.7
CD30	Activated B and T cells, cells of anaplastic large cell lymphoma and more weakly on cells of some cases of other types of large cell lymphoma; Hodgkin cells and Reed–Sternberg cells
CD94	Expressed on a large proportion of normal NK cells and cytotoxic T cells
CD161	Expressed on a large proportion of normal NK cells and cytotoxic T cells
CD158a, CD158b and CD158e	One or other expressed on normal NK cells and cytotoxic T cells

NK, natural killer.

Table 6.17 Characteristic immunophenotype of chronic T-cell leukaemias.

Marker	LGLL – T cell	LGLL – NK cell	T-PLL	ATLL	Sézary syndrome
ERFC/CD2	++	++	++	++	++
CD3	++	–	+	++	++
CD5	–/+	–/+	++	++	++
CD7	–/+	–/+	++	–/+	–/+
CD4	–	–	++	++	++
CD8	++	–/+	–/+	–	–
CD25	–	–	–/+	++	–/+

The frequency with which a marker is positive in >30% of cells in a particular leukaemia is indicated as follows: ++, 80–100%, +, 40–80%; –/+, 10–40%; –, 0–9%.
ATLL, adult T-cell leukaemia/lymphoma; ERFC, E rosette-forming cells; LGLL, large granular lymphocyte leukaemia; NK, natural killer; T-PLL, T-cell prolymphocytic leukaemia.

use of antibodies to the variable (V) domains of TCRβ chains has the potential to indicate clonality in around 60% of mature T-cell neoplasms [248] and CD158 (killer inhibitory receptor, KIR) antibodies can also be useful but neither is widely used. Clonality of T-lineage or NK-lineage cells may be inferred when a cell population shows a uniform, often aberrant, immunophenotype. However, definitive demonstration of clonality requires specialized techniques. In the case of T-lineage leukaemias, this may be either DNA analysis, to show rearrangement at one or more of the *TCR* loci, or cytogenetic analysis. For leukaemias of NK-cell lineage, usually only cytogenetic analysis is applicable but not all cases will have a clonal cytogenetic abnormality.

With advances in immunophenotyping and cytogenetic and molecular genetic analysis it is now possible to recognize many specific entities among T-cell and NK-cell neoplasms. Of the 18 entities recognized in the 2008 WHO classification, four usually present with disseminated disease including leukaemia. Circulating neoplastic cells are also, by definition, present in Sézary syndrome. These conditions will be discussed in detail with conditions that do not usually have a leukaemic presentation being dealt with more briefly.

T-cell large granular lymphocyte leukaemia

Large granular lymphocyte leukaemia can be of T or NK lineage. A definitive diagnosis of the former can be made without difficulty since T-lineage can be demonstrated and, if necessary, clonality can be established by analysis of *TCR* loci. Diagnosis of the latter is more difficult.

Clinical, haematological and cytological features

T-cell large granular lymphocyte leukaemia occurs predominantly in the elderly [249,250]. About a third of patients are asymptomatic at the time of diagnosis [251,252]. Symptomatic patients usually present either with recurrent infection, resulting from neutropenia, or with signs and symptoms of anaemia. There is a strong association with Felty syndrome (rheumatoid arthritis with neutropenia and splenomegaly) and a less strong association with other autoimmune diseases [249] such as pure red cell aplasia and amegakaryocytic thrombocytopenia. Cyclical thrombocytopenia, attributable to cyclical disappearance of megakaryocytes from the bone marrow, has also been described [253]. Lymphadenopathy is uncommon, but hepatomegaly and splenomegaly are frequent findings. Skin lesions are present in less than 20% of patients [254]. The disease typically has a prolonged survival with an actuarial median survival, in one series, of 166 months [252]. In a minority of patients, particularly those whose cells coexpress CD3 and CD56, the disease has a more aggressive clinical course, similar to that of aggressive NK-cell leukaemia [255]; this variant has been referred to as NK-cell-like T-cell lymphoma [256]. Rarely, spontaneous remission occurs [257]. Pregnancy may be associated with both a reduction in the lymphocytosis and an improvement in associated neutropenia [258].

Most patients have an increased WBC, lymphocytosis and an increase in large granular lymphocytes (LGL) [251,259]. Sometimes the WBC is not increased although there is an increase in the number of LGL and sometimes there is no increase in LGL although the LGL are clonal [260]. Lymphocytosis may appear only after splenectomy or with disease progression. The neoplastic cells are usually morphologically very similar to normal LGL (Fig. 6.37). Usually leukaemic cells have a round or oval nucleus with moderately condensed chromatin; the cytoplasm is voluminous and weakly basophilic and contains fine or coarse azurophilic granules. Smear cells are rare. In a minority of patients, cells are small rather than large or granules are very infrequent although the cases, in other ways, are typical of the disease. In NK-cell-like T-cell lymphoma cells are larger and more pleomorphic (Fig. 6.38). Neutropenia is sometimes cyclical. Some patients have isolated neutropenia or thrombocytopenia or, less often, anaemia. These cytopenias are out of proportion to the degree of bone marrow infiltration and appear to have an immune basis. Anaemia may be due to pure red cell aplasia or to a Coombs-positive or Coombs-negative haemolytic anaemia [249]. Macrocytosis is sometimes present. Depending on the nature of the anaemia, the reticulocyte count may be either very low or increased. In some patients cytopenia is attributable to hypersplenism.

The bone marrow shows a variable degree of infiltration by cells with the same morphology as those in the blood. In the early stages, infiltration

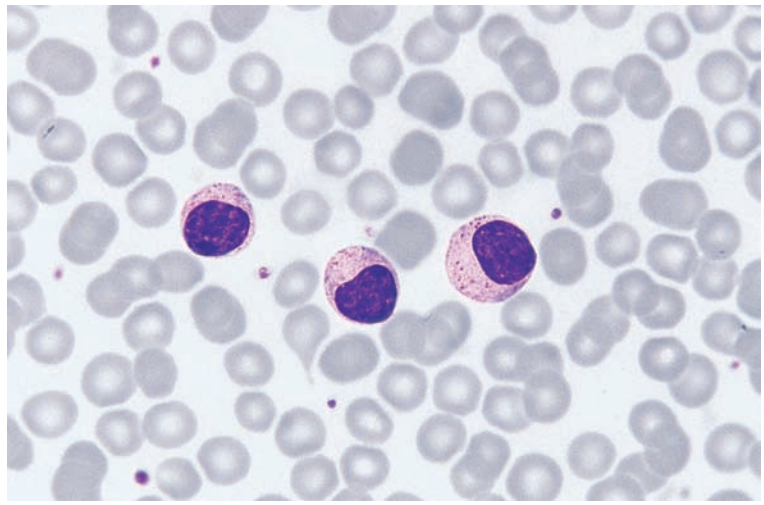

Fig. 6.37 PB film in large granular lymphocyte leukaemia. The cells have abundant weakly basophilic cytoplasm containing prominent azurophilic granules. MGG ×100.

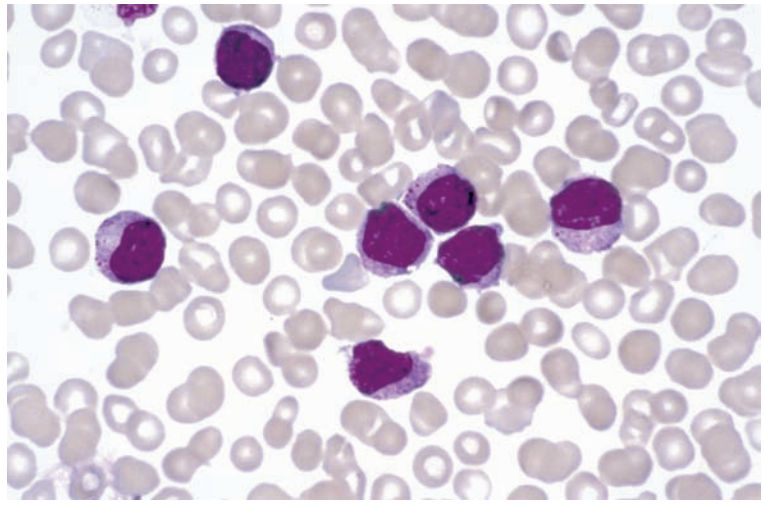

Fig. 6.38 PB film in natural killer-like large granular T-cell lymphoma. The cells are larger and more pleomorphic than in the typical form of large granular T-cell leukaemia and expressed not only CD3 but also four NK markers: CD11b, CD16, CD56 and CD57. (By courtesy of Dr Ayed Eden and the *British Journal of Haematology*.)

may be undetectable or minimal. However, in some patients without an absolute peripheral blood lymphocytosis, examination of the bone marrow may be important for diagnosis. In cases complicated by immunologically mediated cytopenia there may be pure red cell aplasia, megaloblastic erythropoiesis or apparent arrest of granulocyte maturation. Patients with thrombocytopenia usually have normal or increased numbers of megakaryocytes but one case of amegakaryocytic thrombocytopenia has been reported (associated with pure red cell aplasia) [249].

Rheumatoid factor and antinuclear antibodies are often detectable and there is usually a polyclonal increase in immunoglobulins [249].

Immunophenotype

The immunophenotype resembles that of a normal large granular T lymphocyte but differs from it in significant respects [45,261–263]. The most characteristic phenotype is CD2, CD3, CD8, CD57 and TCRαβ positive. Expression of CD2, CD3 and CD8 may be weaker than normal and expression of CD57 may be stronger. CD5 and CD7 may be less

strongly expressed than on normal T lymphocytes or expression may be totally lacking [263,264]. There is no expression of TdT or CD1 and not usually of CD4 (see Table 6.17). In a minority of patients there is expression of CD4 but not CD8 [265]. The frequency of expression of CD16 has varied between different series of patients, possibly reflecting the specific antibody used for its detection, but the majority of cases appear to be positive. CD11b, CD56 and HLA-DR are sometimes positive. TCRαβ is expressed in the majority of patients with TCRγδ being expressed in a minority [265]. CD94 and CD161, both expressed on only a minority of normal T cells, are expressed in around half of patients [263]. There is expression of cytotoxic granule constituents, e.g. perforin, TIA-1, granzyme B and granzyme M. The KIR antigens recognized by antibodies of the CD158 cluster are expressed in 50–70% of patients but because of the clonal nature of the cells there is usually homogeneous expression of only one molecule, either CD158a, CD158b, CD158e, CD158i or CD158k [263,266]; occasionally two KIR molecules are expressed [266]. In one study CD26, a co-stimulatory molecule with CD45 for T-cell activation, was not expressed [264] whereas in another study CD26 was expressed in 6 of 9 patients, expression correlating with severe neutropenia and susceptibility to infection [267]. One case has also been reported showing a mixed T and B immunophenotype [268].

Histology

A trephine biopsy is not usually diagnostically very useful since the specific cytological features of LGL cannot be discerned. Cellularity may be increased, normal or decreased. Bone marrow infiltration may be undetectable or minimal. Immunohistochemistry is important in the detection of a minor degree of infiltration. When detectable, infiltration is usually random focal or interstitial but is sometimes diffuse and occasional patients have shown nodular infiltration [269–271]. There may also be intravascular infiltration, both sinusoidal and intracapillary [263], the latter leading to a linear array of neoplastic cells. Lymphoid nodules may be reactive, containing B cells and CD8-positive T cells and lacking cytolytic T cells [263]. Some cases have plasmacytosis [271] and there may be reactive lymphoid nodules composed of B cells and non-clonal T cells [272]. Patients with red cell aplasia show few erythroid cells beyond the proerythroblast stage. In patients with neutropenia and 'maturation arrest' there are increased numbers of apoptotic cells [273]. Megakaryocytes are virtually absent in patients with amegakaryocytic thrombocytopenic purpura [274]. A significant minority of patients show trilineage myelodysplasia. Reticulin deposition is often increased [272].

Splenic infiltration is in the red pulp, sometimes with an associated plasma cell infiltrate [134].

Cytogenetic and molecular genetic features

A number of clonal cytogenetic abnormalities have been described in T-cell large granular lymphocytic leukaemia but no consistent association has been recognized; T-cell receptor genes may have been involved in translocations in two patients and three reported patients have had complex chromosomal abnormalities (defined as at least three unrelated abnormalities) [275]. Rearrangement at *TCR* loci is usually demonstrable. This is most often rearrangement of *TCRB* and *TCRG* loci but occasionally it is the *TCRG* locus alone. Gene expression microarray analysis has revealed six genes that are expressed in T-cell large granular lymphocyte leukaemia but not in normal LGL [276].

Chronic lymphoproliferative disorders of NK cells

Lymphoproliferative disorders of NK lineage may be either clinically aggressive or clinically indolent. In the absence of good markers of NK cell clonality, recognition of clinically indolent or chronic cases as neoplastic is difficult. However, the aberrant phenotypes expressed and the clonality that can be demonstrated in some cases by analysis of X-linked polymorphisms suggest that clinically indolent disease represents NK-cell large granular lymphocyte leukaemia, at least in some patients [263]. An abnormal but uniform immunophenotype can also be regarded as a surrogate marker of clonality and in some patients clonality can be surmised from the pattern of expression of CD158 epitopes [263].

In the 2008 WHO classification this is a provisional entity, designated chronic lymphoproliferative disorders of NK cells, which requires the presence of at least 2×10^9/l NK cells persisting for

more than 6 months and not being explained by any primary disease [277]. There are sometimes associated autoimmune conditions and neutropenia [249,278]. Hepatomegaly and splenomegaly are rare and the condition shows little tendency to progress [278]. Cytologically the neoplastic cells resemble normal LGL. Bone marrow infiltration is interstitial and intrasinusoidal [277]. Neoplastic cells do not express CD3 but may express cytoplasmic CD3ε [277]. In a series of 11 patients with non-aggressive NK-cell lymphocytosis, there was expression of CD16 and variable expression of other NK-cell-associated antigens; there was expression of CD56 in 45% of patients, of CD57 in 60%, of CD94 in 91% and of CD161 in 40%. A third of patients expressed antigens of the CD158 cluster but there was expression of only CD158a or CD158b or CD158e, providing evidence of clonality [263]. CD158 expression may be lacking.

Problems and pitfalls

Certainty that an individual patient with chronic NK-cell lymphocytosis has a neoplastic condition may be difficult to achieve. This may represent a heterogeneous group of disorders rather than a specific entity. Table 6.18 compares the clinicopathological features of chronic lymphoproliferative disorder of NK cells with other conditions that also express markers associated with cytotoxic T cells and NK cells; these conditions need to be distinguished from each other.

Aggressive NK-cell leukaemia

The WHO classification recognizes an aggressive leukaemia of NK lineage [279]. This condition is more common in the Far East (mainland China, Hong Kong, Taiwan and Japan) than in the West. In almost all cases the neoplastic cells show evidence of infection with EBV [280,281].

Clinical, haematological and cytological features

The frequency of aggressive NK-cell leukaemia is about one sixth that of T-cell large granular lymphocyte leukaemia. Patients are typically younger and often have hepatosplenomegaly and B symptoms (weight loss, fever and night sweats) [249]. Gastrointestinal and central nervous system infiltration may occur. The disease shows aggressive clinical behaviour [282,283], is highly resistant to therapy and has a poor prognosis with many patients surviving less than 2 months [249,281]. Death is usually from multiorgan failure with coagulopathy [249] and sometimes a haemophagocytic syndrome.

The peripheral blood shows a variable increase in LGL [281]. Leukaemic cells resemble normal LGL but, in comparison with the cells of T-cell large granular lymphocyte leukaemia, they are often atypical – larger with more basophilic cytoplasm, hyperchromatic or diffuse chromatin, nuclear irregularity and sometimes nucleoli [249,281] (Fig. 6.39a). There may be circulating nucleated red blood cells and myelocytes. Anaemia and thrombocytopenia are frequent findings but severe neutropenia is less common than in T-cell large granular lymphocyte leukaemia [249].

The bone marrow aspirate shows a variable degree of infiltration by cells similar to those in the peripheral blood (Fig. 6.39b). Increased macrophages and haemophagocytosis are often prominent [281,284].

Immunophenotype

The immunophenotype resembles that of a normal NK cell (see Table 6.17) but differs in significant respects with expression of various markers often being weaker or stronger than normal. Leukaemic cells are CD2 positive but CD3, TCRαβ and TCRγδ negative; CD4 is negative and CD8 may be weakly positive or negative. There is usually expression of cytoplasmic CD3ε, CD11b, CD16 or CD56 and sometimes CD57; CD7 and activation markers such as CD38 and HLA-DR may be expressed [45,261,285,286].

Histology

On trephine biopsy histology there is a variable degree of bone marrow infiltration; the pattern of infiltration may be diffuse, interstitial or angiocentric. There is a monomorphic infiltrate of medium-sized cells with round nuclei and condensed chromatin [281]. The neoplastic cells express CD56 and are negative for CD3 and CD4 but the presence of CD3ε may mean that they appear to be CD3 positive if polyclonal antisera are used. Some patients show haemophagocytosis. Bone marrow fibrosis has been reported [249].

Table 6.18 A comparison of the clinicopathological features of T-cell large granular lymphocyte leukaemia, chronic lymphoproliferative disorder of NK cells, aggressive NK-cell leukaemia and blastic plasmacytoid dendritic cell neoplasm (previously known as blastic NK cell lymphoma).

Disease	Cytology	Epidemiology	Rheumatoid arthritis and other autoimmune phenomena	Usual immunophenotype	Clinical course
T-cell large granular lymphocyte leukaemia	Large granular lymphocytes	No EBV association	Yes	CD2+ CD3+ CD4–, CD8+ CD57+, TCRαβ+, CD11b, CD16 and CD56 variable*, granzyme B+, perforin+ and TIA-1+	Indolent*
Chronic lymphoproliferative disorder of NK cells	Large granular lymphocytes	No EBV association	Possible	CD3– CD16+ CD56 weak; variable expression of CD2, CD7 and CD57, granzyme B+, granzyme M and TIA-1+	Indolent
Aggressive NK-cell leukaemia	Atypical large granular lymphocytes	Strong EBV association; much commoner in Far East Asian populations	No	CD2+ CD3– CD4–, CD8 weak or negative, CD56+ TCRαβ–, CD11b and CD16 variable, CD57–, granzyme B+, perforin+ and TIA-1+	Aggressive
Blastic plasmacytoid dendritic cell neoplasm	Blastic cells, with or without granules	No EBV association	No	CD2– CD3– CD4+, CD8– CD56+, TCRαβ–, CD11b– CD16– CD57–, granzyme B–, perforin– and TIA-1–, TdT and CD34 sometimes positive	Aggressive

EBV, Epstein–Barr virus; NK, natural killer; TIA-1, T-cell intracellular antigen 1; TCR, T-cell receptor; TdT, terminal deoxynucleotidyl transferase.
* Cases of T-cell large granular lymphocyte leukaemia showing expression of CD56 tend to have larger, more pleomorphic neoplastic cells and a more aggressive clinical course; they have been referred to as NK-like T-cell large granular lymphocyte leukaemia.

Cytogenetic and molecular genetic features

Many cases show clonal cytogenetic abnormalities. An association with duplication of 1q, rearrangements of 3q, del(6q), del(11q), –Y, –10 and –13 have been reported [179,287]. *TCR* loci are not rearranged. In most cases, EBV early RNA (EBER) can be detected by *in situ* hybridization [281] with a single episomal form being present [279].

T-cell prolymphocytic leukaemia

The FAB group proposed the term T-cell prolymphocytic leukaemia (T-PLL) for a group of cases that had cells showing cytological similarities to B-PLL, together with other cases that differ cytologically but have the same immunophenotypic and molecular features (see below).

The condition described as 'Sézary cell-like leukaemia' appears to be a variant of T-PLL.

Clinical, haematological and cytological features

T-cell prolymphocytic leukaemia is rare. It is mainly a disease of the elderly and is slightly more common in males [254]. Ataxia telangiectasia predisposes to the development of this type of leukaemia. Cases of

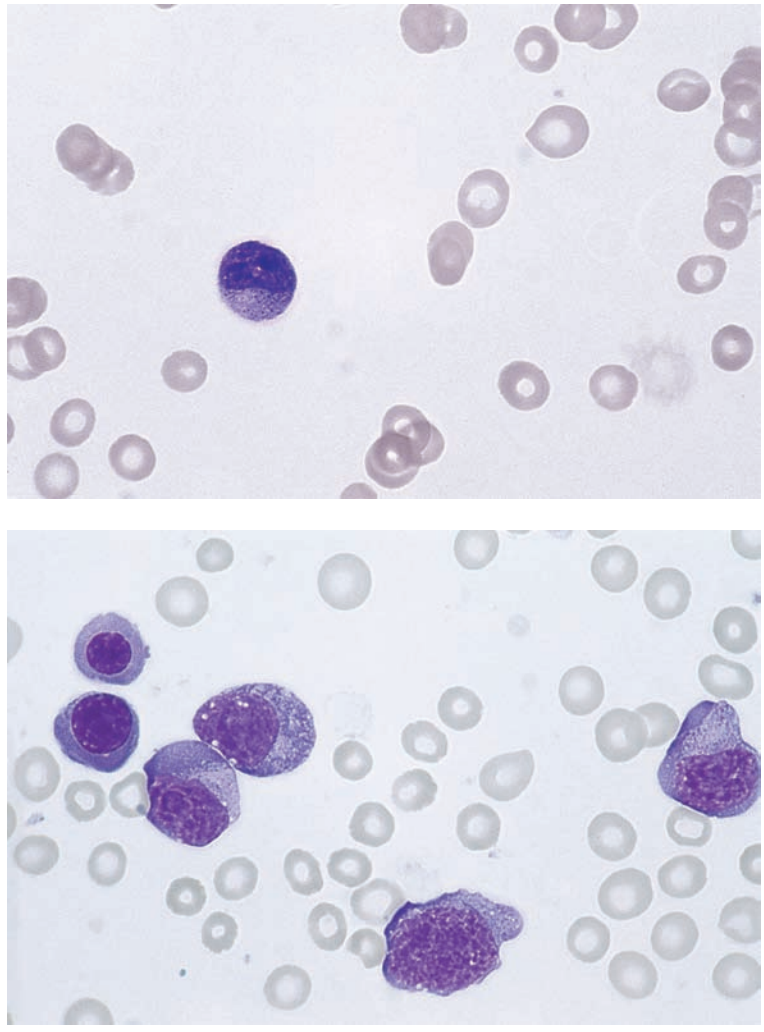

Fig. 6.39 PB and BM in aggressive NK cell leukaemia; the PB (a) shows a medium-sized lymphoid cell with azurophilic granules while the BM (b) shows infiltration by medium-sized, granular lymphoid cells, some of which are nucleolated. MGG ×100. (By courtesy of Dr A. Martin Noya, Seville.)

T-PLL resemble B-PLL in most often presenting with marked splenomegaly and a high WBC [4,288,289]. They differ in that hepatomegaly, lymphadenopathy, skin infiltration and serous effusions may also be present. Fever and central nervous system infiltration sometimes occur. A small minority of patients are asymptomatic and the diagnosis is made incidentally. In most patients the clinical course is aggressive but in about a third the disease is more indolent [290]. Sometimes the disease course is biphasic with a period of disease stability being followed by rapid progression [291]. A single patient has been reported in whom a slow and complete spontaneous remission occurred [292].

The WBC is moderately to greatly elevated and in about a third of patients there is anaemia and thrombocytopenia [254]. In about 50% of patients, morphology of the leukaemic cells is similar to that in B-PLL although the nuclear outline may be more irregular. There may be a minor population of cells with polylobated nuclei or of Sézary-like cells. Ultrastructural examination shows the prominent nucleolus very clearly (Fig. 6.40). In other patients cells are smaller with a higher nucleocytoplasmic ratio and a less readily detectable nucleolus

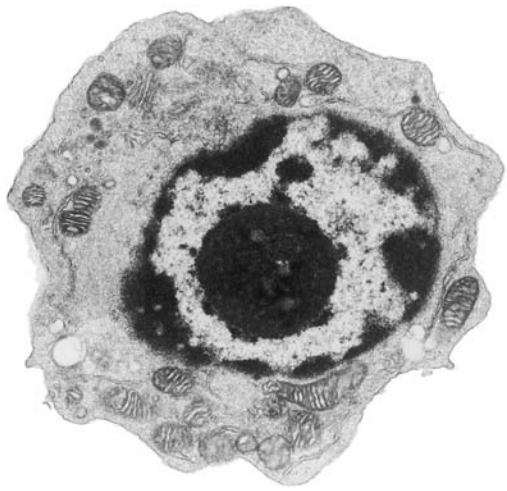

Fig. 6.40 Ultrastructural examination in T-cell prolymphocytic leukaemia (T-PLL) showing a regular nuclear outline, a prominent nucleolus and relatively abundant cytoplasm with a well-developed Golgi zone, rough endoplasmic reticulum and numerous mitochondria. (By courtesy of Dr Estella Matutes, London.)

(Fig. 6.41). Cytoplasm is usually deeply basophilic. In about a quarter of patients the cells are small and the nucleolus is not easily detectable on light microscopy; the scanty cytoplasm is basophilic and may form blebs. Such cases have a prominent nucleolus on ultrastructural examination, have the same clinical course as other patients and show the same cytogenetic abnormality and immunophenotypic features. It has been suggested that the presence of cytoplasmic blebs is the result of cell shrinkage [78] but nevertheless this is a common feature that therefore suggests this diagnosis. In about 5% of patients, cells have cerebriform nuclei [293]. In a small minority of patients multilobulated cells ('flower cells') dominate [290].

Immunophenotype

In most cases of T-PLL there is expression of CD2, CD3, CD5, CD7, CD4 and TCRαβ and CD8 is not expressed [4] (see Table 6.17). In the remaining cases neoplastic cells are negative for CD4 and positive for CD8 (15% of patients) or coexpress these two markers (25% of patients). Coexpression of CD4 and CD8 is otherwise uncommon in mature-T-cell neoplasms. CD7 is expressed more strongly than on normal T lymphocytes and CD3 less strongly [264]; about 20% of cases fail to express surface membrane CD3 and TCRαβ [254]. CD7 positivity helps to distinguish T-PLL from other disorders of mature T cells (Fig. 6.42). CD52 expression is usually strong [293]. In about a fifth of patients there is failure of expression of CD26 [265]. A minority of cases are CD25 positive with expression being weak. A significant minority of cases express CD117 [294]. Some cases, particularly those with aggressive disease, express CD38 [290]. The phenotype CD45R0+ CD45RA– also correlates with aggressive disease [290].

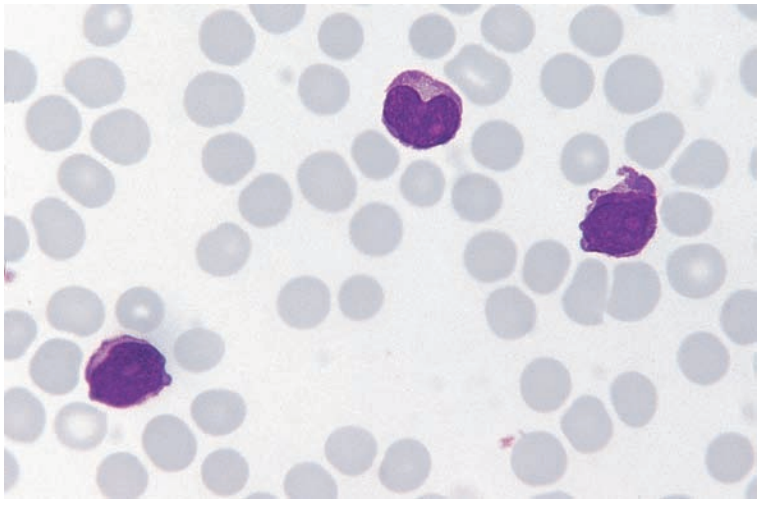

Fig. 6.41 PB film in T-PLL. In this case the nuclei are more irregular and the nucleoli are less conspicuous than in B-PLL. MGG ×100.

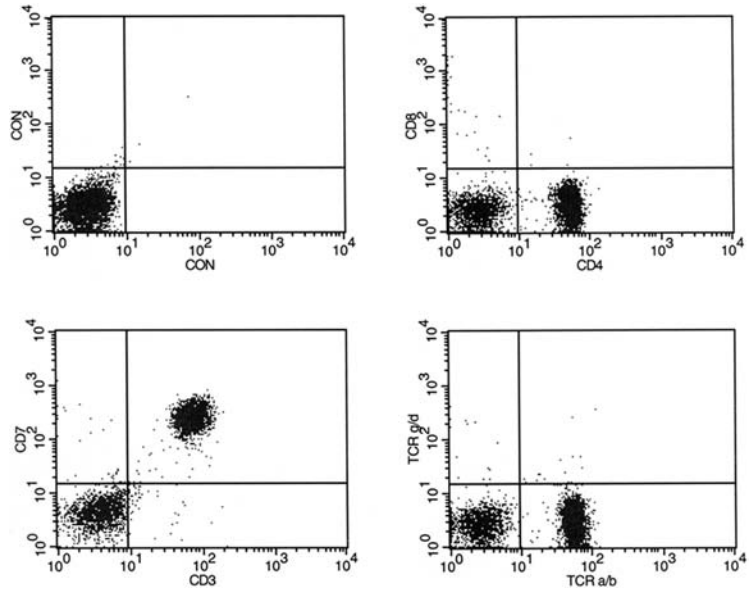

Fig. 6.42 Flow cytometric immunophenotyping in T-PLL showing characteristic positivity for CD3, CD4 and CD7. T-cell receptor (TCR) αβ was expressed but TCRγδ was not. CON, control. (By courtesy of Dr Estella Matutes and Mr Ricardo Morilla.)

Histology

Bone marrow infiltration is usually interstitial or diffuse, although an interstitial/nodular pattern has also been reported. Lymph node infiltration is diffuse and preferentially paracortical. High endothelial venules may be prominent. Splenic infiltration is primarily in the red pulp but extends into the atrophic white pulp [293]. Cutaneous infiltration is in the dermis, particularly around vessels and skin appendages. *TP53* may be overexpressed. Nuclear and cytoplasmic *TCL1* expression can be detected by immunohistochemistry in the majority of patients and can be used for the detection of residual disease after treatment [265,295]. Strong expression of *TCL1* and *AKT* is prognostically adverse [293].

Cytogenetic and molecular genetic features

About three quarters of cases of T-PLL show either inv(14)(q11q32) or t(14;14)(q11;q32) [82,296]. These chromosomal rearrangements involve the *TCRAD* locus at 14q11 and two oncogenes, *TCL1A* and *TCL1B*, at 14q32.1. *TCL1A* and *TCL1B* are dysregulated and, when overexpressed, inhibit apoptosis. t(X;14)(q28;q11) is a less common translocation in which the *MTCP1* gene at Xq28 is brought into proximity to the *TCRAD* locus. Dysregulation of *MTCP1* can also result from proximity of this gene to the *TCRB* locus as a result of t(X;7)(q28;q35) [297]. A further uncommon but recurring translocation is t(11;14)(q21;q32) [297]. Other common cytogenetic abnormalities include trisomy 8, add(8p), idic(8)(p11) – previously interpreted as i(8)(q10) – and t(8;8)(p11-12;q12) (all giving rise to trisomy or polysomy of 8q), deletions of the short arm of chromosome 12 [298], deletions of the long arms of chromosomes 6 and 11 and translocations with a breakpoint at 11q23, the site of the *ATM* gene (the gene that is mutated in ataxia telangiectasia). Mutations and deletions of the *ATM* gene are common and may coexist with the characteristic translocations involving *TCL1A* [299]. Deletions of 12p13 occur and lead to haploinsufficiency of *CDKN1B* [300]. Abnormalities of chromosome 17 involving *TP53* occur in about a quarter of patients [293].

FISH analysis, using whole chromosome paints for chromosomes 8 and 14, can be useful in elucidating the nature of complex karyotypes. Probes for the chromosome 8 centromere and for *MYC* can be useful in demonstrating idic(8)(p11).

Problems and pitfalls

Integration of cytological, immunophenotypic and genetic features makes diagnosis straightforward, as long as the possibility of this diagnosis is considered.

Adult T-cell leukaemia/lymphoma

Adult T-cell leukaemia/lymphoma (ATLL) [4,301, 302] occurs in adults who are carriers of the retrovirus, human T-cell lymphotropic virus I (HTLV-I); serology for HTLV-I is positive and proviral DNA is clonally integrated into the DNA of the neoplastic cells. It is somewhat more common in men. Distribution of the disease relates to areas where the virus is endemic. Cases were first recognized in Japan, and subsequently in the Caribbean, in the southern United States and in West Indian immigrants to the UK. Significant numbers of cases have been reported from Eastern Europe, the Middle East, Central and West Africa, South America (Brazil, Chile, Colombia), Taiwan, Australia (aboriginal population) and a number of other countries.

Smouldering and chronic forms of ATLL should also be recognized; suggested criteria are shown in Table 6.19 [303]. Disease progression is not inevitable in patients with smouldering or chronic ATLL. A significant minority of patients with chronic ATLL revert to smouldering ATLL or even to the carrier state and, similarly, a significant minority of patients with smouldering ATLL return to the carrier state [304]. A preleukaemic stage of ATLL in which there is monoclonal proliferation of T lymphocytes is recognized, with about 40% of such individuals progressing to ATLL [305].

Rare cases of ATLL have been related to HTLV-II rather than HTLV-I [295].

Clinical, haematological and cytological features

The disease mainly occurs in adults above the age of 40 years but cases in infants have been described in South America. Most patients present with lymphadenopathy, bone and skin lesions, hypercalcaemia and circulating lymphoma cells. Some patients have hepatomegaly or splenomegaly or infiltration of other organs. In about 10% of patients presentation is as a lymphoma with no abnormal cells in the peripheral blood. Immune deficiency is common so that patients may have opportunistic fungal infections, including *Pneumocystis jirovecii* pneumonia, or hyperinfection with *Strongyloides stercoralis*. Prognosis is generally poor but some patients have a more chronic or smouldering disease. A minority of patients suffer from other HTLV-I-related disorders such as HTLV-I-associated myelopathy and uveitis.

Table 6.19 Subclassification of adult T-cell leukaemia/lymphoma (ATLL) [303].

Category	Peripheral blood lymphocytes	Tissue infiltration	Biochemistry
Smouldering ATLL	Lymphocyte count $<4 \times 10^9$/l and either 5% abnormal lymphocytes or histological proof of lung or skin infiltration	The lungs or skin may be infiltrated but there is no infiltration of lymph nodes, liver, spleen, gastrointestinal tract or CNS and no ascites or pleural effusion	LDH up to 1.5× the upper limit of normal. No hypercalcaemia
Chronic ATLL	Lymphocyte count $>4 \times 10^9$/l and T lymphocytes $>3.5 \times 10^9$/l with morphologically abnormal cells and occasional frank ATLL cells (such as flower cells); in most cases there are >5% abnormal lymphocytes	The lungs, skin, lymph nodes, liver or spleen may be infiltrated, but there is no infiltration of gastrointestinal tract or CNS and no ascites or pleural effusion	LDH up to twice the upper limit of normal. No hypercalcaemia
Lymphoma-type ATLL	Lymphocyte count $<4 \times 10^9$/l and ≤1% abnormal lymphocytes	Histologically demonstrated lymphoma	There may be elevated LDH or hypercalcaemia
Acute, ATLL		All other cases	

CNS, central nervous system; LDH, lactate dehydrogenase.

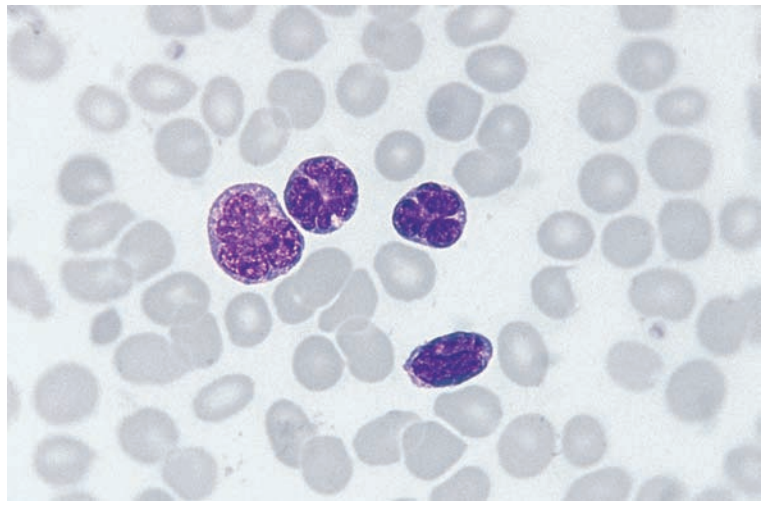

Fig. 6.43 PB film in adult T-cell leukaemia/lymphoma. Cells are pleomorphic with polylobulated nuclei, one of which resembles a clover leaf. MGG ×100.

The number of circulating lymphoma cells is very variable. Their number does not correlate with the degree of bone marrow infiltration. The morphology is distinctive (Fig. 6.43). Cells vary greatly in size and form. Most cells have condensed and relatively homogeneous nuclear chromatin with nucleoli being infrequent and small. A minority of cells are blastic with basophilic cytoplasm. Some cells have convoluted nuclei, and may resemble Sézary cells, while many are polylobated, with some nuclei resembling cloverleaves or flowers. Some cases have atypical cytological features – LGL in one case [306] and bizarre giant cells resembling Hodgkin cells in others [307]. Cells in serous effusions have similar characteristics (Fig. 6.44).

Some patients are anaemic and some have neutrophilia or eosinophilia. The neutrophilia may be marked, e.g. a neutrophil count of $16–55 \times 10^9$/l, and in some cases is attributable to secretion of granulocyte colony-stimulating factor (G-CSF) by neoplastic cells [308].

There is a correlation between cytological features and aggressiveness of the disease. Acute ATLL has a higher percentage of both typical cells with convoluted nuclei and cells with unusual cytological features (lymphoblastoid, vacuolated, granular pleomorphic or large) whereas the proportion of CLL-like cells is lower [309]. Among chronic cases, a higher proportion of CLL-like cells is indicative of a better prognosis [309]. Occasional cases of ATLL have been treated by bone marrow transplantation and in one patient transplanted from an HTLV-I-positive donor, molecular analysis showed that apparent recurrence of leukaemia 4 months after transplantation was of donor origin [310].

Immunophenotype

Adult T-cell leukaemia/lymphoma cells usually express CD2, CD3, CD5, CD4, CD25 and HLA-DR [4,45] (see Table 6.17). A minority of cases are positive for CD7 or CD8 (CD4– CD8+ or CD4+ CD8+). Expression of CD3 is considerably weaker than on normal T lymphocytes and CD7, when expressed, is also weaker than on normal cells [264]. Positivity for CD25 helps to distinguish ATLL from other T-cell disorders, which are usually CD25 negative. However, it should be noted that CD25 can be expressed on a large proportion of T cells in HTLV-I-positive individuals without ATLL. Other activation markers, such as CD38, may be expressed. An atypical immunophenotype, i.e. other than CD4+ CD8–, correlates with a worse prognosis [311].

Histology

The bone marrow may initially be normal or show interstitial infiltration but, with disease progression, diffuse infiltration may be seen. Increased bone destruction by osteoclasts may be apparent. In one patient the pathological features of osteitis fibrosa cystica were seen [312]. Lymph nodes show diffuse infiltration, either paracortical or effacing nodal architecture. Neoplastic cells are very pleomorphic

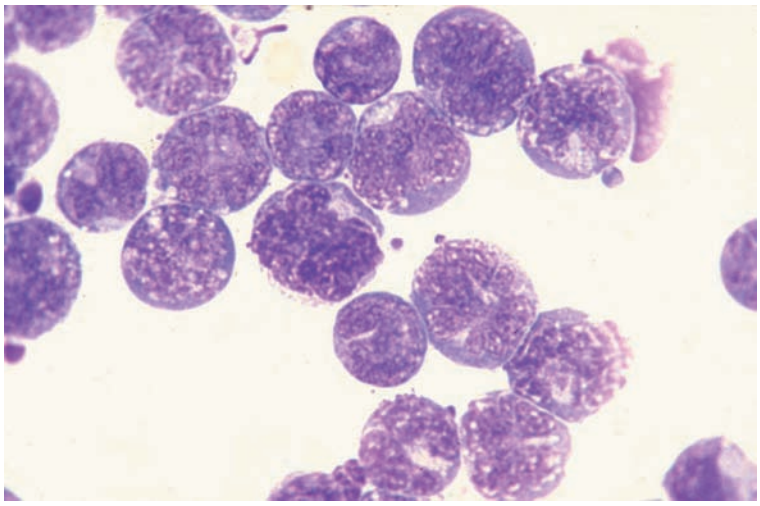

Fig. 6.44 Ascitic fluid of a patient with adult T-cell leukaemia/lymphoma showing pleomorphic cells with lobulated nuclei and basophilic cytoplasm. MGG ×100.

and may include multinucleated giant cells. Infiltration in the skin is perivascular or diffuse in the middle and upper dermis; some cases show epidermotropism with formation of intraepidermal lymphoid infiltrates known as Pautrier microabscesses, formerly thought to be confined to the Sézary syndrome. Immunohistochemistry typically shows cells to express CD3, CD4, CD25 and sometimes CD30 [313] but not CD7.

Cytogenetic and molecular genetic features

The karyotype is usually abnormal. A variety of chromosomal abnormalities have been described in ATLL, most commonly trisomy 3, trisomy 7, trisomy 12, trisomy 21, del(6q) and rearrangements with breakpoints at 7p14-15, 14q11-13 or 14q32 [314]. There may be loss of heterozygosity at 6q15-21. Rearrangements of 1p32-36 and 5q11-13 may also be preferentially associated with ATLL. Complex karyotypes and clonal evolution are common.

Mutations of tumour suppressing genes, *CDKN2A* (p16), *CDKN2B* (p15) and *TP53*, may be found in the acute and lymphomatous forms of ATLL.

HTLV-I is clonally integrated in leukaemic cells at a random site, which differs between patients. Multiple integration in different clones is associated with indolent disease whereas multiple integration in a single clone is associated with aggressive disease [315]. Integration of defective HTLV-I is also associated with more aggressive disease [316].

Problems and pitfalls

Diagnosis from cytology and immunophenotype is usually straightforward. However, the diagnosis can be missed on lymph node histology if the pathologist is not aware that the patient falls into a risk group for HTLV-I infection. The presence of intrapidermal lymphoma cells is a recognized feature and should not lead to misdiagnosis of Sézary syndrome.

Mycosis fungoides and Sézary syndrome

Sézary syndrome and mycosis fungoides are related cutaneous T-cell lymphomas. Sézary syndrome can be viewed as an erythrodermic variant of mycosis fungoides but in the WHO-EORTC (European Organisation for Research and Treatment of Cancer) classification of primary cutaneous lymphomas they are separate entities [317–319]. Mycosis fungoides usually runs a chronic course but transformation to an aggressive large cell lymphoma, including anaplastic large cell lymphoma, can occur. The clinical course of Sézary syndrome is much shorter. Prognostic factors in mycosis fungoides include advanced stage, older age, higher LDH and histological transformation [318].

Clinical, haematological and cytological features

Sézary syndrome is characterized by circulating lymphoma cells together with pruritis and a generalized exfoliative or infiltrative erythroderma, the

cutaneous manifestations being consequent on infiltration. Mycosis fungoides may be restricted to the skin with the cutaneous infiltrate presenting clinically as patches, plaques or, eventually, tumours; the tumours may ulcerate. When the disease becomes more generalized, lymphoma cells identical to those in Sézary syndrome may circulate in the blood.

Sézary cells [4] may be either large or small and one or other form usually predominates in an individual patient. Large Sézary cells are similar in size to a neutrophil or a monocyte with a high nucleocytoplasmic ratio and a round or oval nucleus with densely condensed chromatin, a grooved surface and usually no detectable nucleolus (Fig. 6.45). Small Sézary cells are similar in size to a normal small lymphocyte with a high nucleocytoplasmic ratio and a dense hyperchromatic nucleus with a grooved surface and no visible nucleolus (Fig. 6.46). Some cells show a ring of cytoplasmic vacuoles; periodic acid–Schiff (PAS) staining shows this to be due to the presence of glycogen. One patient has been described in whom Sézary cells had cytoplasmic projections [320]. Small Sézary cells, in particular, may be difficult to identify on light microscopy. Electron microscopy can be very useful since it reveals the highly complex, convoluted nucleus (Fig. 6.47). Not surprisingly, an increasing number of circulating lymphoma cells correlates with worse prognosis in patients with erythrodermic cutaneous T-cell lymphoma [321]. The lymphocyte count and the percentage of Sézary cells have been found to be predictive of response to extracorporeal photopheresis [322].

The Hb and platelet count are usually normal. There may be a reactive eosinophilia.

Immunophenotype

The characteristic immunophenotype of Sézary cells is expression of CD2, CD3, CD4 and CD5 with usually no expression of CD7, CD8 or CD25 [4,323] (see Table 6.17). Both CD8 [317] and CD25 [324] are expressed in a minority of patients. CD3 and CD4 are often more weakly expressed than in normal T lymphocytes. CD2 expression may be weak or lost and both CD3 and CD5 may be expressed abnormally strongly [295]. A half to three quarters of cases are CD7 negative [254]. TCRαβ is expressed [265]. Lack of expression of CD26 is usual [265] whereas about three quarters of normal CD4-positive T cells express CD26. CD103 (cutaneous lymphocyte antigen) is expressed [325].

Mycosis fungoides also usually shows expression of CD2, CD3, CD4, CD5 and CD103 with usually no expression of CD7, CD8 or CD25.

Various immunophenotypic criteria have been proposed for the identification of Sézary cells. These include: (i) the bimodal distribution of CD3 expression when there are both normal and neoplastic cells present [326]; (ii) expression of CD158k [327];

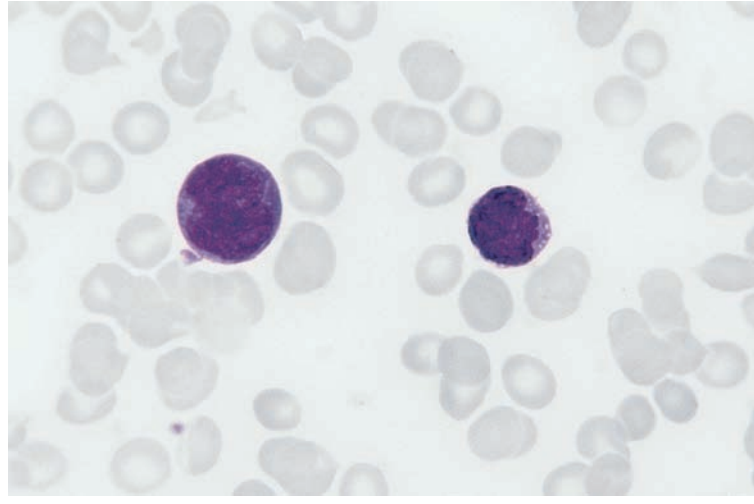

Fig. 6.45 PB film in large cell variant of Sézary syndrome. Both cells have convoluted nuclei; the smaller one has inconspicuous cytoplasmic vacuoles. MGG ×100.

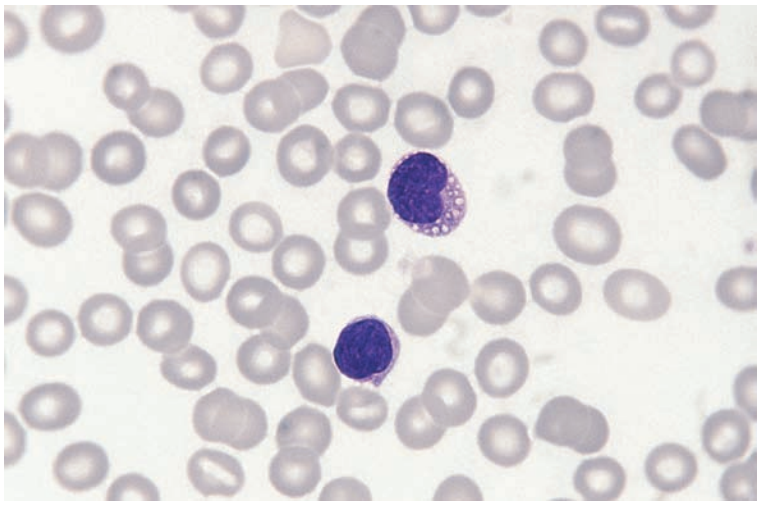

Fig. 6.46 PB film in small cell variant of Sézary syndrome. The nuclei show surface grooves and one cell has a partial circle of vacuoles around the nucleus. MGG ×100.

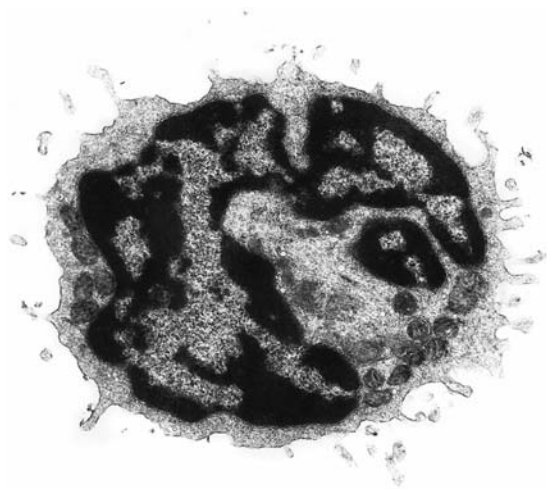

Fig. 6.47 Ultrastructural examination in Sézary syndrome showing the characteristic highly irregular nuclear outline. (By courtesy of Dr Estella Matutes, from reference 8.)

and (iii) absent of weak expression of CD26, usually associated with weak expression of CD4 [328]. Detection of more than 11% of CD4+ CD7− cells by flow cytometry has been found useful in detecting haematological involvement in mycosis fungoides and in confirming the diagnosis of Sézary syndrome in erythrodermic patients [323,324]. Diagnostic criteria for peripheral blood involvement, incorporating immunophenotyping and other data, have been proposed by the International Society for Cutaneous Lymphomas [328]. These are summarized in Table 6.20 [329].

Histology

Bone marrow infiltration is absent in the early stages and, even with advanced disease, is often minimal; infiltration is interstitial. Skin infiltration is in the upper dermis, particularly around the skin appendages, and within the epidermis, forming Pautrier microabscesses in some but not all cases. Epidermotropism and Pautrier microabscesses are characteristic of Sézary syndrome and mycosis fungoides but are not always present and are not pathognomonic since they have now also been observed in a number of cases of ATLL. Epidermotropism in less consistently present in Sézary syndrome than in mycosis fungoides and histological features can be non-specific. In the later stages of mycosis fungoides, when there is tumour formation, epidermotropism may be lost with tumour in both upper and lower dermis. Lymph node infiltration may be paracortical or lymph nodes may be effaced. CD45RO is expressed [327].

Cytogenetic and molecular genetic features

A great variety of cytogenetic abnormalities have been reported in Sézary syndrome without any consistent association being apparent. Random heteroploidy occurs. Clonal abnormalities are often highly complex and polyploid cells are not uncommon. Indirect evidence of a clonal cytogenetic

Table 6.20 International Society for Cutaneous Lymphomas criteria for diagnosis of Sézary syndrome [329].

Type of criterion	Criterion
Morphology	Cells consistent with Sézary cells (defined as lymphocytes with moderately or highly grooved or infolded nuclei) at least 1×10^9/l *with additional evidence of cutaneous T-cell lymphoma*
Immunophenotyping	CD4 : CD8 = >10 : 1 as a result of increased CD3+ CD4+ lymphocytes
	Aberrant lack of expression of pan-T markers CD2, CD3, CD4 or CD5
	CD4+ CD7– lymphocytes ≥40%
Cytogenetic analysis	A chromosomally abnormal T-cell clone
Molecular genetic evidence	Increased lymphocyte count plus evidence of T-cell clonality on Southern blot or polymerase chain reaction

abnormality based on the detection of aneuploidy by flow cytometry has also been found useful in diagnosis [330].

No specific molecular genetic abnormality has been associated with this type of lymphoma. However, demonstration of rearrangement at the *TCR* locus is useful in confirming the diagnosis and has been found to be more sensitive in the detection of peripheral blood involvement than morphometric analysis [325]. The *JUNB* oncogene may be amplified in Sézary syndrome and there may be inactivation of *TP53* and *CDKN2A* [319].

Problems and pitfalls

Since it can be difficult to distinguish the small cell variant of Sézary syndrome from reactive erythrodermic conditions it has been suggested that the detection of a clonal cytogenetic abnormality or demonstration of *TCR* rearrangement should be a prerequisite for the diagnosis of this form of cutaneous T-cell lymphoma [325]. Alternatively, other criteria proposed by the International Society for Cutaneous Lymphomas (Table 6.20) can be applied.

A variant of T-PLL, Sézary cell-like leukaemia, is recognized in which the circulating cells resemble Sézary cells but there are no cutaneous lesions [254,331,332]. Consideration of the immunophenotype (CD7 expression) and the cytogenetic features permit the correct diagnosis. There are usually complex cytogenetic abnormalities including inv(14)(q11q32) and possibly idic(8)(p11) [331].

Hepatosplenic T-cell lymphoma

This is a rare, clinically aggressive T-cell lymphoma in which the neoplastic cells typically express TCRγδ, normally expressed on a minor subset of peripheral blood T lymphocytes, but do not express TCRαβ. Incidence is increased following renal transplantation [333] and in other immunosuppressed patients [334].

Clinical, haematological and cytological features

Patients are relatively young and typically present with widespread disease and systemic symptoms. Hepatosplenomegaly is characteristic but there is usually no lymphadenopathy. Anaemia and thrombocytopenia are common. In one series of patients, a variable degree of peripheral blood involvement was usually detectable [335] (Fig. 6.48). Cytologically, the neoplastic cells cannot be readily distinguished from those of other T-cell lymphomas. The bone marrow is hypercellular with erythroid and megakaryocytic hyperplasia. There is a scanty to moderate infiltrate of small to medium-sized cells with the smaller cells having condensed chromatin while the larger cells are more blastic with a small but easily detectable nucleolus. Some cells have fine granules [335]. There may be increased plasma cells and reactive haemophagocytosis.

Immunophenotype

Characteristically there is expression of CD2, CD3, CD7, CD11b and CD56 [335,336]. CD4, CD5 and CD8 are usually not expressed. CD16 is often expressed whereas CD57 is not [179]. In the majority of patients there is expression of TCRγδ but in a minority there is expression of TCRαβ or coexpression of TCRαβ and TCRγδ. The cytotoxic granule proteins TIA-1 and granzyme M are expressed but not granzyme B or perforin, this being the immunophenotype of a non-activated cytotoxic cell [334].

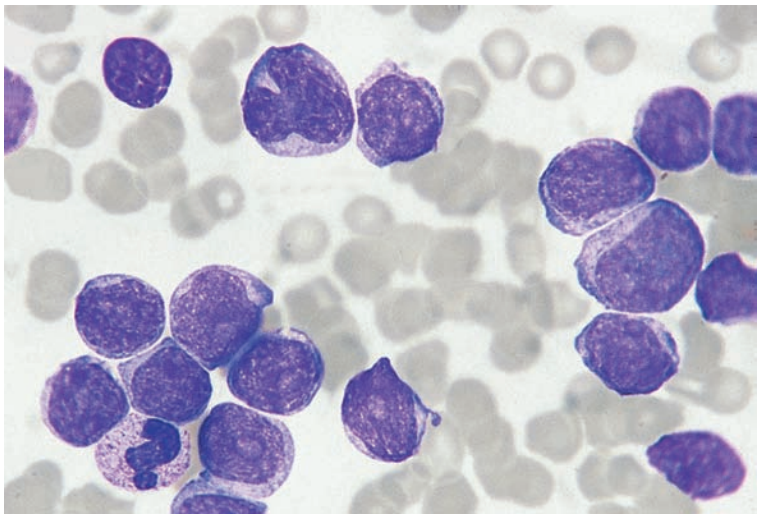

Fig. 6.48 PB film from a patient with hepatosplenic lymphoma in leukaemic phase showing large pleomorphic cells with basophilic cytoplasm. MGG ×100.

Histology

Early in the disease course, bone marrow infiltration is interstitial and intrasinusoidal; the characteristic intravascular pattern of infiltration may suggest this diagnosis (Fig. 6.49). With advancing disease, there is a more extensive interstitial infiltration. There is typically hyperplasia of myeloid cells.

Cytogenetic and molecular genetic features

The most characteristic cytogenetic abnormalities are trisomy 8 and an isochromosome of 7q, i(7)(q10). The majority of cases show a monoclonal rearrangement of *TCRG*, but not of *TCRB*.

Other T-lineage non-Hodgkin lymphoma

Rarely leukaemia occurs as a manifestation of T-cell lymphomas, either at presentation or during the course of the illness.

Clinical, haematological and cytological features

Clinical features may include hepatomegaly, splenomegaly and lymphadenopathy. The blood count may show anaemia or thrombocytopenia.

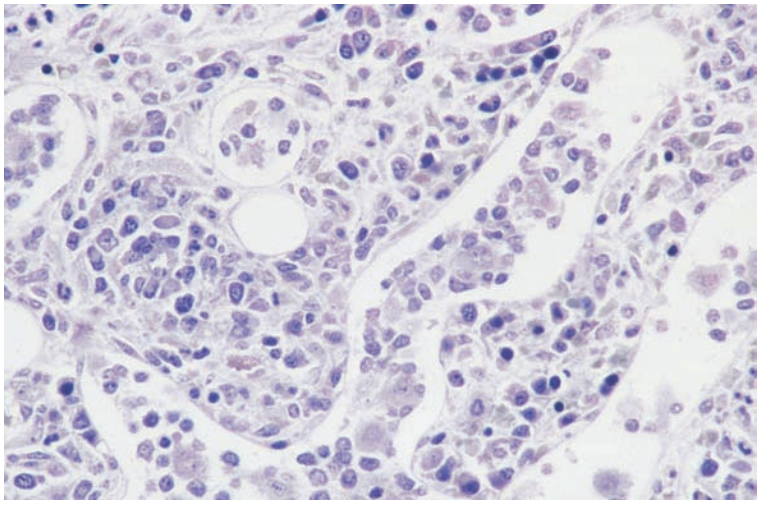

Fig. 6.49 Trephine biopsy section from a patient with hepatosplenic lymphoma in leukaemic phase showing intravascular lymphoma (same patient as Fig. 6.48). Giemsa ×40.

The circulating cells may be small or medium sized with a variable degree of pleomorphism. In other patients neoplastic cells are large, usually with basophilic cytoplasm, prominent nucleoli and considerable pleomorphism (Fig. 6.50).

In some patients with a leukaemic phase of anaplastic large cell lymphoma, lymphoma cells are very large and pleomorphic (Fig. 6.51). However, the small cell variant is more common among cases in leukaemic phase, with lymphoma cells being mainly small to medium sized with only a minority of large cells [337–339]. There is an irregular, hyperchromatic nucleus, which can be lobulated, cerebriform or clover leafed; azurophilic cytoplasmic granules are sometimes present. There may be occasional large cells that resemble Reed–Sternberg cells [339]. Hyperleucocytosis can occur. Bone marrow infiltration may be minimal but cells are distinctive (Fig. 6.52).

The neoplastic cells of T-cell lymphomas cannot generally be distinguished from those of B-lineage lymphomas on the basis of cytological features [233] so that immunophenotyping is essential for precise diagnosis.

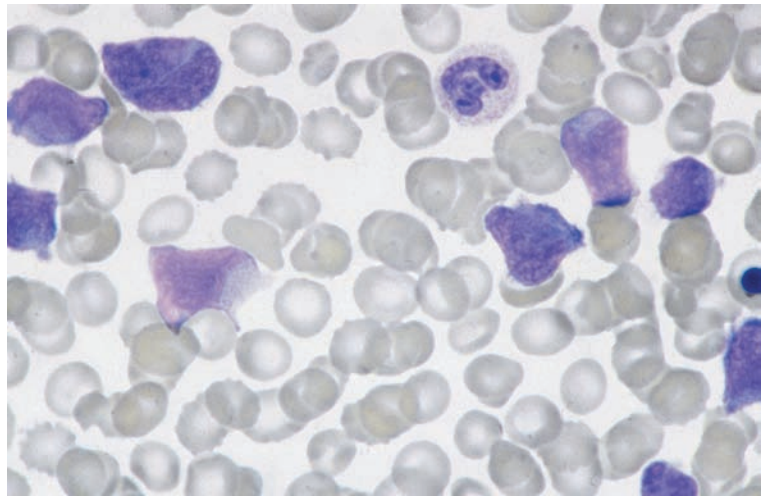

Fig. 6.50 PB film in T-lineage lymphoma. MGG ×100.

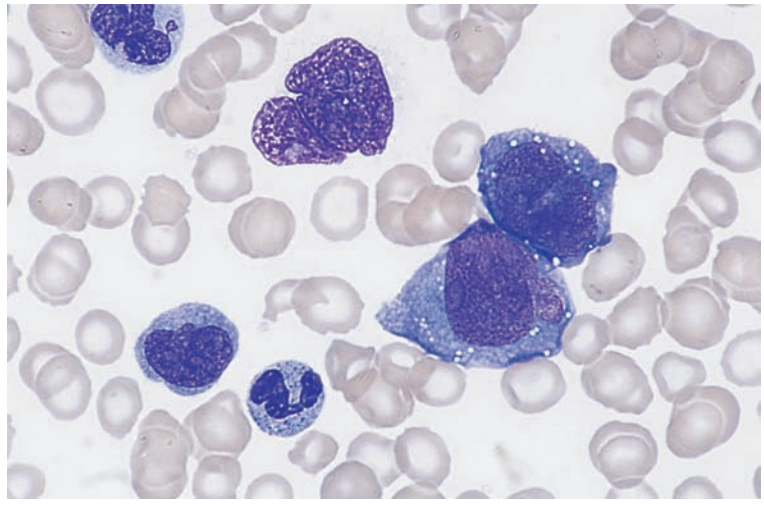

Fig. 6.51 PB in anaplastic large cell lymphoma. The lymphoma cells are very large and very pleomorphic. MGG ×100. (By courtesy of Dr David Clark, Grantham.)

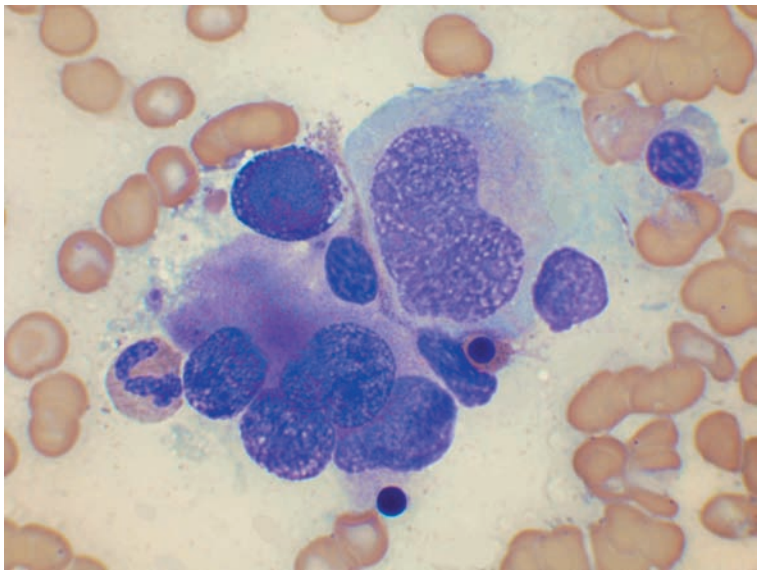

Fig. 6.52 BM aspirate in anaplastic large cell lymphoma. The lymphoma cell (top right) is as large as the megakaryocyte (bottom left). MGG ×100.

Immunophenotype

Although the immunophenotype is that of a mature T cell there is no consistent pattern. Immunophenotypes rarely seen in normal peripheral blood T cells are common [233]. Abnormalities that may occur include under- or overexpression or lack of expression of CD3, underexpression or absent expression of CD7, overexpression of CD5, under- or overexpression of CD2 and failure to express either CD4 or CD8 [340].

A specific immunophenotype is recognizable when there is peripheral blood involvement by anaplastic large cell lymphoma; these cells sometimes express T-lineage markers such as CD2, CD7, CD45RO and either CD4 or CD8, but less often CD3 or CD5 [339]. In addition they usually express CD30, ALK1 (not normally expressed by haemopoietic or lymphoid cells) and EMA [339], this being more readily demonstrable on immunohistochemistry of trephine biopsy sections. CD38, CD56 and HLA-DR may be expressed, but not CD34. Myeloid antigens such as CD11b, CD13, CD15 and CD33 may be expressed [179,339].

Peripheral blood involvement by angioimmunoblastic T-cell lymphoma is characterized by expression of CD10 in addition to CD3 (may be weak), CD4 and, usually, CD2 and CD5.

Histology

In patients with peripheral blood involvement by large cell lymphoma, lymph node biopsies usually show diffuse effacement by lymphoma cells. Bone marrow trephine biopsy sections usually show extensive infiltration by large, highly abnormal cells; the pattern of infiltration is random focal or diffuse. Anaplastic large cell lymphoma can be distinguished from other large cell lymphomas by the typical immunophenotype and the cytological features (very large, highly abnormal cells, often with a cohesive growth pattern).

Cytogenetic and molecular genetic features

A range of cytogenetic and molecular genetic abnormalities is seen, depending on the precise type of lymphoma.

Many patients with anaplastic large cell lymphoma have a specific cytogenetic abnormality, t(2;5)(p23;q35) leading to *NPM1-ALK* fusion. The fusion transcript can be detected by RT-PCR and abnormal ALK expression by immunohistochemistry [184]. In a minority of patients there are other translocations and other fusion genes involving *ALK*. Rearrangements of *ALK* in typical and variant translocations can be detected by dual-colour, break-apart FISH.

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APPENDIX

LEUKAEMIA DIAGNOSIS IN RESOURCE-POOR COUNTRIES

No country has unlimited resources to apply to medical care but problems are particularly acute in developing countries and conflict zones. Laboratories in resource-poor countries need to develop policies that permit them to make optimal use of what is available to them. Health care will often necessarily be focused on major health problems such as malaria, tuberculosis and the acquired immune deficiency syndrome (AIDS) due to infection by the human immunodeficiency virus (HIV). Leukaemia diagnosis and treatment will not be a high priority. Laboratories must therefore seek to develop cost-effective diagnostic protocols for the conditions for which treatment is available. Accurate diagnosis can be particularly important in the developing world so that scarce resources are not wasted on inappropriate treatment. Which particular haematological neoplasms can be treated effectively will depend on the ability to recognize the condition, availability of skilled nursing and medical care, the existence of an adequate blood transfusion service and availability and affordability of relevant drugs. Cost-effective diagnosis will often involve low technology solutions, and both aid donors and developing countries must be aware of the possible pitfalls of introducing instruments that are expensive to maintain and operate and that require a highly trained workforce. Maintaining high standards, with good quality control, for basic tests such as blood counts, blood films and cytochemistry is of critical importance. However modern technology is not necessarily inappropriate. It might appear at first sight that molecular genetic analysis would not be suitable for use in leukaemia diagnosis in a resource-poor country. However, since imatinib is now available free of charge to some developing countries, the introduction of a molecular technique to detect the *BCR-ABL1* fusion gene may well be indicated to confirm the diagnosis of chronic granulocytic leukaemia (CGL). Laboratories involved in leukaemia diagnosis need to be aware of facilities and skills already available in other parts of their health service that could have an expanded application. For example, a flow cytometer and the expertise to operate it might already be in use in HIV management and skills in molecular techniques might already have been developed for identification of microorganisms.

Some developing countries have arrangements for their citizens with certain leukaemias to be treated in another country; for example, patients from New Caledonia are referred to Australia and patients from Portuguese-speaking African countries are sometimes transferred to Portugal. In these circumstances the clinical and laboratory recognition of a probable leukaemia becomes important even when local treatment facilities are not available. Other countries, e.g. in the Middle East and North Africa, may lack health service infrastructure but do not lack financial resources. The best solution, at least in the short term, may then be outsourcing some of the more complex investigations, e.g. cytogenetic analysis, to a laboratory in another country.

Considerable improvements in outcome of childhood leukaemia have followed twinning of institutions in developing countries with institutions in developed countries. Twinning between specialist centres and peripheral centres within the same developing countries can also be useful. Such collaboration may involve establishing treatment protocols as well as diagnostic methods. The development of

regional immunophenotyping laboratories serving more than one country with advice and support from a major centre in a developed country can be cost effective. Collaborative schemes for acute leukaemia diagnosis have been established between a US centre and El Salvador, Honduras and Guatemala, between another US centre and India and between Nicaragua, Italy and Switzerland. A 2010 conference on acute promyelocytic leukaemia will specifically discuss treatment of this condition in the developing world. The initial step in such collaborations may be that some of the diagnostic procedures are carried out in a developed country but progress should be towards setting up an appropriate diagnostic service in the developing country.

Haematologists may well have to concern themselves with efficient methods of transporting specimens if networking within and beyond a country is to be effective; imaginative solutions may be found, e.g. ultralight unmanned aircraft for transport of small specimens within a country has been pioneered in South Africa. A more conventional combination of postage and e-mail can give a turn around time averaging about 2 weeks. Consultation with experts may also be possible through the internet and e-mail (tele-haematology). If a digital camera fitted to a microscope is not available it is possible to use a mobile phone that includes a camera to capture an image without the need for any special adoptions; the captured image can be transmitted through the mobile phone network or by e-mail.

If sending blood and bone marrow films for an expert opinion careful labelling with the patient's name, the date and the nature of the material (peripheral blood or bone marrow) is important. Full clinical details and the results of the blood count must be supplied plus a provisional diagnosis and a statement as to the diagnostic problem that requires advice.

In planning diagnostic tests it needs to be remembered that in some developing countries patients need to pay for tests so that there may be serious financial constraints affecting diagnosis as well as treatment.

The rest of this appendix will deal first with cost-effective techniques for the diagnosis of specific leukaemias and then with training and continuing education of laboratory medical and scientific staff.

Diagnosis of specific leukaemias and related conditions

Diagnosis of acute leukaemia

Morphology of peripheral blood and bone marrow films is critical. Many leukaemias can be recognized from clinical context, blood count and morphological features alone. It is thus possible to recognize, with a high degree of reliability, acute myeloid leukaemia (AML) belonging to the French–American–British (FAB) categories of M2, M3, M4, M5b and M6. By adding (i) either a Sudan black B (SBB) stain or a myeloperoxidase (MPO) stain plus (ii) a non-specific esterase stain such as α-naphthyl acetate esterase it becomes possible to recognize the FAB M1 category and most cases of FAB M5a AML. If a case of acute leukaemia has the cytological features of FAB L1 acute lymphoblastic leukaemia (ALL) it is highly likely that it does represent ALL. This is particularly so if the patient is a child and the likelihood is increased if there is block positivity on a periodic acid–Schiff (PAS) stain. This leaves cases that cannot be readily distinguished by morphology and cytochemistry, specifically the FAB categories of M0 and M7 AML and the L2 category of ALL. Morphological evidence may correctly suggest the lineage; for example, coexisting dysplastic changes in myeloid cells would suggest M0 AML rather than ALL whereas cytoplasmic blebs might indicate M7 AML.

Consideration should be given to the possibility of flow cytometry immunophenotyping for recognizing FAB M0 and M7 AML and L2 ALL and possibly also for the confirmation of cases of FAB L1 ALL. A restricted range of antibodies will permit most cases to be diagnosed, e.g. CD33, anti-MPO and possibly CD41 for the myeloid lineage including megakaryoblasts, CD19 or the cytoplasmic epitope of CD79a for the B lineage and cytoplasmic CD3 for the T lineage. The European LeukemiaNet recommends for the initial diagnosis of acute leukaemia: cytoplasmic MPO, CD117, terminal deoxynucleotidyl transferase (TdT), cytoplasmic CD3, CD7, cytoplasmic CD79a and CD19 (a panel that includes two myeloid, two B-lymphoid and two T-lymphoid markers and a marker of immaturity) but if circumstances dictate this can be reduced. Since it usually possible to recognize that a case of leukaemia represents acute leukaemia by morphology, TdT (and

CD34) may be considered inessential. If it is not possible to provide flow cytometry immunophenotyping, an alternative way to identify the lineage of an acute leukaemia is by immunohistochemistry. If there is otherwise no need for a trephine biopsy, this technique can be applied to sections of a clotted bone marrow aspirate; after films have been spread the rest of the aspirate can be left in the syringe to clot and then teased out and dropped into formalin. After fixation it is sectioned as for any histological specimen, without any need for decalcification. Appropriate antibodies that are likely to be available include CD79a (more appropriate than CD20, which is often negative) for B lineage, CD3 for T lineage and CD61 for megakaryocytes. Assuming that that the cytochemical stains for granulocytic and monocytic lineage cells mentioned above have already been done on a bone marrow aspirate or a peripheral blood film, not much is gained by adding an MPO stain. However, either anti-lysozyme or CD68 could be added for the confirmation of myeloid lineage if cytochemistry were, for any reason, unavailable. Immunohistochemistry for TdT is not necessary if it is apparent from a blood film or bone marrow aspirate film that the patient has some type of acute leukaemia. Immunohistochemistry has the important advantage that sections can be transported to another centre or even another country much more readily than samples for flow cytometric immunophenotyping.

Sometimes consideration of clinical as well as haematological features permits a strong presumptive diagnosis of ALL. Thus, if a patient has an acute leukaemia that has no morphological or cytochemical markers of myeloid differentiation and has a mediastinal mass it is highly probably that the diagnosis is T-lineage ALL and if appropriate confirmatory techniques are not available treatment as such is justified. Likewise a child with cytological features of L1 ALL can legitimately be treated as having ALL if confirmatory techniques are not available. It should be recognized that using nothing but clinical features, morphology and a SBB stain the lineage assignment of a case of acute leukaemia will be right most of the time. The addition of a non-specific esterase stain will help to confirm acute monoblastic leukaemia.

If a definite lineage assignment cannot be made in a case of acute leukaemia, a trial of therapy may help, e.g. chemotherapy appropriate for ALL if the differential diagnosis is between ALL and AML or a trial of all-*trans*-retinoic acid (ATRA) if acute promyelocytic leukaemia is strongly suspected but the diagnosis is not certain.

Cytogenetic analysis may be impossible in a resource-poor country but if imatinib is available and molecular analysis for *BCR-ABL1* has been introduced for confirmation of the diagnosis of CGL then it should also be applied to adults with a confirmed or presumptive diagnosis of ALL.

In the absence of cytogenetic and molecular techniques it is not possible to apply the World Health Organization (WHO) classification of AML fully. In this circumstance, with the use of cytochemistry, cases can be diagnosed and classified as in the FAB classification. However, the WHO cut-off point of 20% rather than 30% blast cells should be used to separate AML from MDS.

Diagnosis of chronic granulocytic leukaemia

Morphology of peripheral blood and a careful differential count will identify patients who are highly likely to have CGL. If imatinib is available, molecular confirmation is more likely to be feasible than cytogenetic analysis and one or other is likely to be required by any pharmaceutical company or charity providing or subsidizing the drug. In other circumstances, a diagnosis based on morphology, differential count and a neutrophil alkaline phosphatase score is usually sound.

Diagnosis of other myeloproliferative neoplasms

When no highly specific treatment is available, cytogenetic and molecular analysis may be considered inessential in the investigation of suspected polycythaemia vera, essential thrombocythaemia and primary myelofibrosis. These conditions (which are not considered in this book) can be diagnosed with reasonable reliability from the blood film, blood count, bone marrow aspirate and trephine biopsy. Molecular diagnosis of the rare examples of chronic eosinophilic and related leukaemias due to rearrangement of *PDGFRA* or *PDGFRB* is unlikely to be feasible in a developing country, particular as other causes of eosinophilia greatly outnumber cases of eosinophilic leukaemia. In a country that lacks the necessary infrastructure rather than

financial resources, outsourcing the investigations or a trial of imatinib may be feasible.

Diagnosis of myelodysplastic syndromes and myelodysplastic/myeloproliferative neoplasms

Diagnosis of the myelodysplastic syndromes (MDS) and the myelodysplastic/myeloproliferative neoplasms (MDS/MPN) such as chronic myelomonocytic leukaemia (CMML) and atypical chronic myeloid leukaemia (aCML) is usually possible from clinical and haematological features. Occasional cases of MDS may not be recognized in the absence of cytogenetic analysis but with follow-up the diagnosis is likely to become apparent. The only type of MDS for which a fairly specific treatment is available is MDS with isolated del(5q) and other cases with the same cytogenetic abnormality. The specific treatment, lenalidomide, is expensive and may not be available in a developing country. If such treatment could be feasible then many, but not all, cases could be selected for cytogenetic analysis or fluorescence *in situ* hybridization (FISH) on the basis of identification of a refractory anaemia with or without ring sideroblasts with the mean cell volume being high in the normal range or elevated and megakaryocytes being hypolobated.

Cases of CMML can usually be recognized without difficulty from the clinical features and the blood count and film. Cases of aCML, however, can be confused with the accelerated phase of CGL and because of the therapeutic implications of the latter diagnosis, analysis for *BCR-ABL1* should therefore be applied if available. The diagnosis of juvenile myelomonocytic leukaemia (JMML) can be difficult and it is important to be aware of the viral infections that can simulate it. The presence of definite dysplasia and observation of an increased haemoglobin F can be useful. The blood film is usually quite sufficient to distinguish JMML from CGL.

Diagnosis of leukaemias and lymphomas of mature lymphocytes

A specific diagnosis of various leukaemias and leukaemic phase lymphomas of mature B, T and NK lymphocytes can often be suspected from clinical and cytological features. If immunophenotyping and cytogenetic/molecular genetic analysis are available, the diagnosis is greatly facilitated. In the absence of easy availability of such techniques, it is necessary to focus on certain diagnoses that have major therapeutic implications. Histolology and immunohistochemistry can be very useful when other techniques are lacking.

Burkitt lymphoma, particularly endemic Burkitt lymphoma, can usually be recognized with high reliability from clinical, cytological and histological features. If a single immunohistochemical stain were to be added for confirmation it would be a stain such as Ki-67 or MIB1 to demonstrate that the proliferating fraction approaches 99%.

Hairy cell leukaemia should be recognized since, even if cladribine and pentostatin are not available, the patient may be effectively treated with interferon or by splenectomy. Cytological features are highly characteristic and monocytopenia is almost invariable. Immunological confirmation is unlikely to be available in a resource-poor setting since four very specific antibodies are needed. A tartrate-resistant acid phosphatase stain is therefore advised. Trephine biopsy histology can also be very characteristic.

Adult T-cell leukaemia/lymphoma (ATLL) has a fairly specific geographical distribution. With the advent of surprisingly effective specific treatments the diagnosis is important. Cytology is often very characteristic and if there is hypercalcaemia the suspicion of this diagnosis is strengthened. Demonstration of seropositivity for the human T-cell lymphotropic virus I (HTLV-I) is essential for the diagnosis, but it must be remembered that seropositive patients can also develop other types of leukaemia or lymphoma. If flow cytometry immunophenotyping is not available, immunohistochemistry is an alternative method for demonstrating a T-cell population that is expressing CD25.

The diagnosis of other lymphomas in leukaemic phase (e.g. mantle cell lymphoma, follicular lymphoma) can be made by histology and immunohistochemistry when circumstances do not permit the diagnosis from peripheral blood cytology, immunophenotyping and molecular genetic analysis. However, it should be noted that, apart from Burkitt lymphoma and ATLL, a precise diagnosis as to the subtype of a lymphoma may not be necessary for selection of treatment as long as it is certain that the patient does have a lymphoma and it is clear whether it is high grade or low grade. If rituximab is available then it becomes important to distinguish

Table A1 Websites giving useful information in the diagnosis of leukaemia and related conditions.

Organization	Web address
American Society of Hematology	http://www.hematology.org/
European Hematology Association	http://www.ehaweb.org/
European LeukemiaNet (site includes an atlas and information on cytogenetics and immunophenotyping)	http://www.leukemia-net.org/content/home/
British Society for Haematology (includes a small image atlas)	http://www.b-s-h.org.uk/
BloodMed (educational site of the British Society for Haematology with Wiley-Blackwell; includes a large image atlas; access for 1 year is available free of charge)	www.bloodmed.com
British Committee for Standards in Haematology	http://www.bcshguidelines.com/
Atlas of Genetics and Cytogenetics in Haematology and Oncology	http://atlasgeneticsoncology.org/
Bloodline.net (includes a small image atlas)	http://www.bloodline.net/
University of Utah WebPath (includes a haematopathology image atlas)	http://library.med.utah.edu/WebPath/webpath.html

diffuse large B-cell lymphoma from T-cell lymphomas and this is likely to be most efficiently done by immunohistochemistry for the detection of CD20 expression. In some countries the providers of rituximab are willing to supply kits for assessment of CD20 expression.

The diagnosis of chronic lymphocytic leukaemia is often reliable when based on characteristic clinical and cytological features. However, if immunophenotyping is not available, recognition of atypical cases and their distinction from non-Hodgkin lymphoma may not be possible. In a resource-poor country, if the most appropriate drugs for treatment are not available, the lack of a precise diagnosis may not necessarily have any adverse effect on the management of the patient.

Training and continuing education

When resources are limited it is of critical importance that laboratory scientific and medical staff acquire and maintain morphological skills.

There are some educational aids that are available at low cost or free of charge to developing countries. Table A1 shows some of the websites that give access (free or partly free) to information on various aspects of haematology, including leukaemia diagnosis; the usefulness of these sites is not, of course, confined to resource-poor countries. Books and bench aids are available to developing countries at a reduced cost from the World Health Organization (WHO). The UK charity, Teaching AIDS at Low Cost (TALC), http://www.talcuk.org/, provides low price books to developing countries. Many medical journals are available free of charge to the world's poorest countries.

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