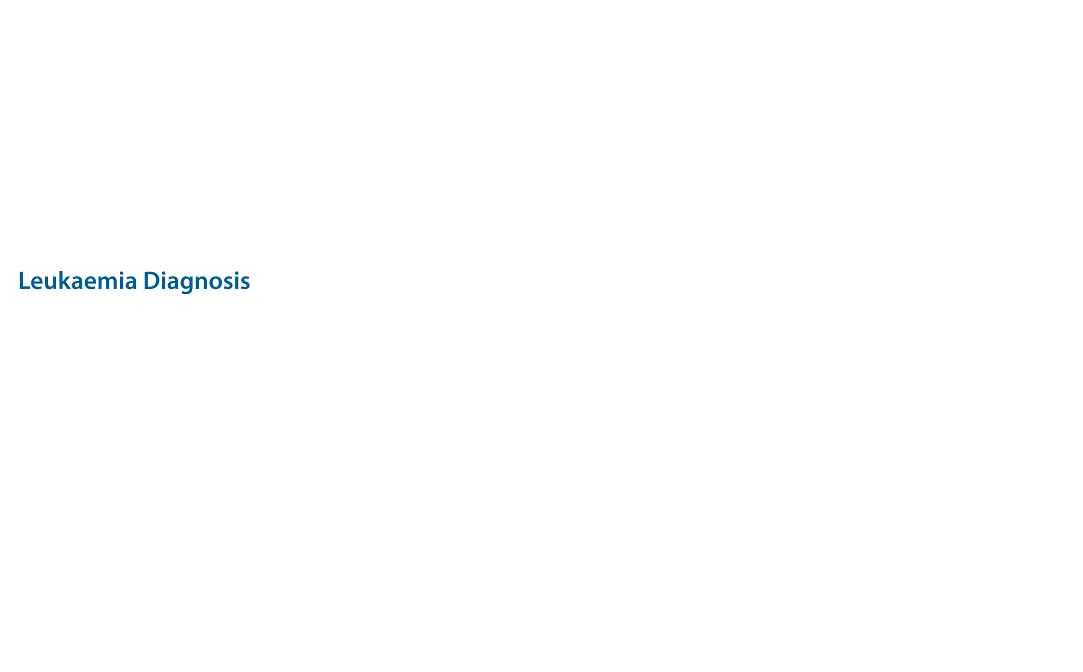

Leukaemia Diagnosis

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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 and 2016 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. A previous appendix has now become a chapter, dealing with the diagnosis of leukaemia in under-resourced laboratories. This may seem presumptuous from someone who has not worked in a developing country for more than 50 years but I thought that at least it should be attempted.

Since photographs have been taken using 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. Dr Dora Mbanya from Cameroon and Dr Amma Benneh from Ghana kindly reviewed Chapter 8. My thanks are also due to the considerable number of friends and colleagues from five continents who helped by lending material for photography or supplying photographs; they are individually acknowledged in the relevant figure legends.

Barbara J. Bain, 2017

Commonly Used Abbreviations

Specific cytogenetic abbreviations are shown in Table 2.10.

aCML	atypical chronic myeloid leukaemia
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
ANBE	α-naphthyl butyrate esterase
APAAP	alkaline phosphatase–anti-alkaline phosphatase (technique)
APC	allophycocyanin (a fluorochrome)
APL	acute promyelocytic leukaemia
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
CHIP	clonal haematopoiesis of indeterminate potential
cIg	cytoplasmic immunoglobulin
CLL	chronic lymphocytic leukaemia
CLL/PL	chronic lymphocytic leukaemia, mixed cell type with prolymphocytoid cells
CML	chronic myeloid or myelogenous leukaemia
CMML	chronic myelomonocytic leukaemia
CNL	chronic neutrophil leukaemia
CT	computed tomography
CyIg	cytoplasmic immunoglobulin
DIC	disseminated intravascular coagulation
DLBCL	diffuse large B-cell lymphoma
DNA	deoxyribonucleic acid
EBV	Epstein–Barr virus
EDTA	ethylenediamine tetra-acetic acid
EGIL	European Group for the Immunological Characterization of Leukemias
EMA	epithelial membrane antigen
ERFC	E-rosette-forming cells
ET	essential thrombocythaemia
FAB	French–American–British
FDG-PET	^{18}F-fluorodeoxyglucose positron emission tomography
FISH	fluorescence *in situ* hybridization
FITC	fluorescein isothiocyanate (a fluorochrome)
FSC	forward light scatter
G6PD	glucose-6-phosphate dehydrogenase

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-1 human T-cell lymphotropic virus 1
ICUS idiopathic cytopenia of undetermined significance
IDUS idiopathic dysplasia of uncertain significance
Ig immunoglobulin
IGH immunoglobulin heavy chain locus
IGHV immunoglobulin heavy chain variable region genes
IPSS International Prognostic Scoring System
ITD internal tandem duplication
JMML juvenile myelomonocytic leukaemia
L1–L3 categories of acute lymphoblastic leukaemia in the FAB classification
LDH lactate dehydrogenase
LGL large granular lymphocyte/s
LUC large unstained (peroxidase-negative) cell
M : E myeloid : erythroid
M0–M7 categories of acute myeloid leukaemia in the FAB classification
MAC morphology–antibody–chromosomes (technique)
MALT mucosa-associated lymphoid tissue
McAb monoclonal antibody/ies
MCV mean cell volume
MDS myelodysplastic syndrome/s
MDS/MPN myelodysplastic/myeloproliferative neoplasm/s
MDS-U myelodysplastic syndrome, unclassifiable
M-FISH multiplex fluorescence *in situ* hybridization
MGG May–Grünwald–Giemsa (a stain)
MIC Morphology, Immunophenotype, Cytogenetics
MIC-M Morphology, Immunophenotype, Cytogenetics, Molecular genetics
MLD multilineage dysplasia
MPAL mixed phenotype acute leukaemia
MPN myeloproliferative neoplasm/s
MPO myeloperoxidase
MRC Medical Research Council
MRD minimal residual disease
MRI magnetic resonance imaging
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
NRBC nucleated red blood cells
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 (a fluorochrome)
PerCP peridinin chlorophyll protein complex (a fluorochrome)
Ph Philadelphia (chromosome)
PLL prolymphocytic leukaemia
PMF primary myelofibrosis
PPO platelet peroxidase
PTD partial tandem duplication
PV polycythaemia vera
RA refractory anaemia
RAEB refractory anaemia with excess of blasts
RAEB-T refractory anaemia with excess of blasts in transformation
RARS refractory anaemia with ring sideroblasts
RARS-T refractory anaemia with ring sideroblasts and thrombocytosis

RC refractory cytopenia
RCC refractory cytopenia of childhood
RCMD refractory cytopenia with multilineage dysplasia
RCUD refractory cytopenia with unilineage dysplasia
RN refractory neutropenia
RNA ribonucleic acid
RQ-PCR real-time quantitative polymerase chain reaction
RS ring sideroblasts
RT refractory thrombocytopenia
RT-PCR reverse transcriptase polymerase chain reaction
SBB Sudan black B
SKY spectral karyotyping
SLD single lineage dysplasia
SLVL splenic lymphoma with villous lymphocytes
SmIg surface membrane immunoglobulin
SMZL splenic marginal zone lymphoma
SNP single nucleotide polymorphism
SSC side scatter of light (flow cytometry term)
TAM transient abnormal myelopoiesis
t-AML therapy-related acute myeloid leukaemia
TCR T-cell receptor
TdT terminal deoxynucleotidyl transferase
TKD tyrosine kinase domain
TKI tyrosine kinase inhibitor
t-MDS therapy-related myelodysplastic syndrome
TRAP tartrate-resistant acid phosphatase
WBC white blood cell count
WHO World Health Organization
WPPS WHO-classification-based Prognostic Scoring System
ZAP70 zeta-associated protein 70

1

The Nature of Leukaemia, Cytology, Cytochemistry and the Morphological Classification of Acute Leukaemia

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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 either normal cellular genes (proto-oncogenes) that have mutated or are dysregulated, or 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 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. Lymphoid leukaemias usually arise in a B- or T-lineage stem cell but occasionally acute lymphoblastic leukaemia (ALL, either B-ALL or T-ALL) arises in a lymphoid-myeloid stem cell, as shown by development of histiocytic sarcoma with the same clonal origin as the preceding B- or T-lineage ALL [1,2].

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 deoxyribonucleic 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 phenotype leukaemias, the latter showing both lymphoid and myeloid differentiation (or both T- and B-lineage 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 aetiology of leukaemia

Many potential causes of leukaemia are known, but nevertheless the majority of cases remain unexplained. There may be an underlying genetic or other constitutional predisposition in addition to oncogenic environmental factors.

There is a familial predisposition to myelodysplastic syndromes (MDS) and acute myeloid leukaemia (AML). In the cases of MDS/AML, predisposing mutations have been identified in a number of genes: *RUNX1*, *CEBPA*, *GATA2*, *ANKRD26*, *SRP72*, *DDX41*, *ETV6*, *ATGB2/GSKIP* (duplication) and possibly *HYDIN*,

MUC16, *NMUR2*, *RNF213* and *ACD* (*TPP1*) [3,4]. Fanconi anaemia, dyskeratosis congenita, Down syndrome, Shwachman–Diamond syndrome, severe congenital neutropenia (with life sustained by treatment with granulocyte colony-stimulating factor) predispose to AML. Down syndrome also predisposes to ALL. Neurofibromatosis, Noonan syndrome and *CBL* mutation-associated syndrome predispose to juvenile myelomonocytic leukaemia. There is a familial predisposition to chronic lymphocytic leukaemia.

Cytotoxic chemotherapy, immunosuppressive therapy and acquired aplastic anaemia predispose to MDS and AML. To a lesser extent, cytotoxic chemotherapy predisposes to ALL and mixed phenotype acute leukaemia (MPAL). Irradiation predisposes also to AML, ALL and chronic myeloid leukaemia (CML).

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 diagnosis and classification of leukaemia is based initially on morphology. A significant advance in the diagnosis and morphological classification of leukaemias occurred with the development of the French–American–British (FAB) classification of acute leukaemia [5–9], and subsequently of other leukaemias and related conditions. This classification, developed by a collaborating group of French, American and British haematologists provided clearly defined criteria, 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 and replaced by the *WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues* [10]. The WHO (World Health Organization) 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 provide a useful shorthand description of morphological subtypes. It is of value in the preliminary evaluation 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.

The nature and classification of acute leukaemia

Acute leukaemia comprises a heterogeneous group of conditions that differ in aetiology, pathogenesis, molecular mechanisms, optimal treatment and prognosis. The heterogeneity is reduced when cases of acute leukaemia are divided into AML, ALL and 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,

Table 1.1 Cytochemical stains of use in the diagnosis and classification of acute leukaemia [11–13].

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)(q24.1;q21.2) and t(8;21)(q22;q22.1)
α-Naphthyl acetate esterase (ANAE) (‘non-specific’ esterase)	Stains monocytes and macrophages, megakaryocytes and platelets, most T lymphocytes and some T lymphoblasts (focal); may be expressed by melanoma cells [13]
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, but see Chapter 8.

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 [11–13].

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 are characterized by ineffective haemopoiesis, that is, there is usually 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 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, or 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 also 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 5.

The nature and classification of chronic myeloid leukaemias and myelodysplastic/myeloproliferative neoplasms

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)(q34.1;q11.2) is variously referred to as 'chronic granulocytic leukaemia', 'chronic myelogenous leukaemia', 'chronic myelogenous leukaemia, *BCR-ABL1* positive' and 'chronic myeloid leukaemia'. The designation chronic myeloid leukaemia will be used in this book since it is the term now favoured by the WHO [10], but it is not an ideal term since it is also used as a generic term and is thus ambiguous.

Chronic myeloid leukaemia is similar in nature to other myeloproliferative neoplasms (MPN) such as polycythaemia vera, essential thrombocythaemia and primary myelofibrosis, with which it is grouped in the WHO classification. In these related conditions differentiation is to erythrocytes in polycythaemia vera, to platelets in essential thrombocythaemia, to all myeloid lineages in primary myelofibrosis, and to neutrophils in chronic neutrophilic leukaemia. The distinguishing features of primary myelofibrosis are extramedullary haemopoiesis and bone marrow fibrosis, which despite the name is not actually 'primary' but is reactive to the myeloid neoplasm. These other MPN can undergo clonal evolution, including evolution to a chronic myeloid leukaemia and blast transformation.

Certain other chronic myeloid leukaemias are associated with specific molecular abnormalities and are classified on this basis. These include cases with mutation of genes encoding proteins on signalling pathways, specifically rearrangement of *PDGFRA*, *PDGFRB* or *FGFR1*, or formation of a *PCM1-JAK2* fusion gene. Such cases are classified on the basis of the molecular abnormality.

Other chronic myeloid leukaemias are more closely related to MDS than to MPN and are thus classified as myelodysplastic/myeloproliferative neoplasms (MDS/MPN). MPN are characterized by effective proliferation of myeloid cells and increased numbers of end cells, whereas MDS is characterized by ineffective proliferation, morphological dysplasia and inadequate numbers of end cells of one or more lineages. When a condition shows effective proliferation of cells of one lineage and ineffective proliferation of cells of another lineage with associated dysplasia it is classified as 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 MDS/MPN are discussed in detail in Chapter 5, and other chronic myeloid leukaemias in Chapter 6.

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.

In some lymphoid neoplasms, the neoplastic cells are 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 designated ALL. Primary infiltration of other lymphoid organs or tissues by lymphoblasts is designated 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 2008/2016 WHO classification, lymphoid precursor neoplasms are designated 'B lymphoblastic leukaemia/lymphoma' and 'T lymphoblastic leukaemia/lymphoma'.

In other lymphoid neoplasms the neoplastic cells are mature, and again 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. Similarly, a rare subtype of mature T-cell neoplasm, designated adult T-cell leukaemia/lymphoma, presents as leukaemia in about 90% of patients and as lymphoma in about 10%. 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 leukaemias 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.

Defining a blast cell, a promyelocyte and a promonocyte

Blast cells are large cells with a high nucleocytoplasmic ratio, often nucleoli and usually a delicate, diffuse chromatin pattern although some lymphoblasts are smaller with some chromatin condensation. 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 [14]. 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 18) rather than granules; less often they contain large rectangular crystals [15] or large inclusions (pseudo-Chédiak–Higashi inclusions). Auer rods and pseudo-Chédiak–Higashi granules may coexist in the same blast cell (Fig. 1.1). Occasionally blast cells contain micronuclei, which may represent acentric chromosomal fragments, damaged single chromosomes or amplified oncogenes [16]. Rarely leukaemic myeloblasts have aberrant condensation of chromatin into large blocks [17].

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 lacking other characteristics of promyelocytes [18]. They have divided blast cells into 'agranular blasts' and 'granular blasts'. This definition of a blast cell has been accepted in the WHO classification.

Cells are categorized as promyelocytes rather than type II/III or granular blasts when they

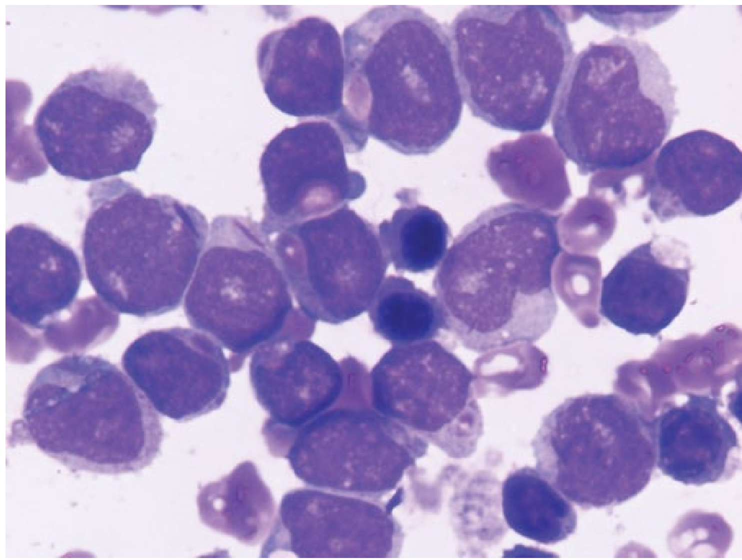
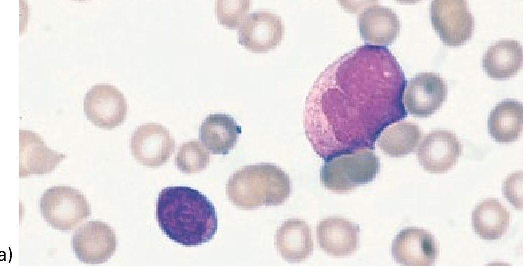


Fig. 1.1 The peripheral blood (PB) film of a patient with acute myeloid leukaemia (AML) showing some blast cells containing Auer rods and others containing pseudo-Chédiak–Higashi granules. May–Grünwald–Giemsa (MGG) × 100. (With thanks to Dr Abbas Hashim Abdulsalam, Baghdad.)

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 type I, II and III blasts and as promyelocytes are shown in Figs 1.2–1.5. 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). Granular blast cells are generally myeloid, but occasionally lymphoblasts have a few myeloperoxidase-negative granules. Rarely lymphoblasts contain inclusions resembling Russell bodies but unrelated to immunoglobulin [19].

Monoblasts (Fig. 1.5a) differ from myeloblasts in being larger with more voluminous cytoplasm. The cytoplasm is moderately to markedly basophilic and may have fine granules or vacuoles.

(a)



(b)

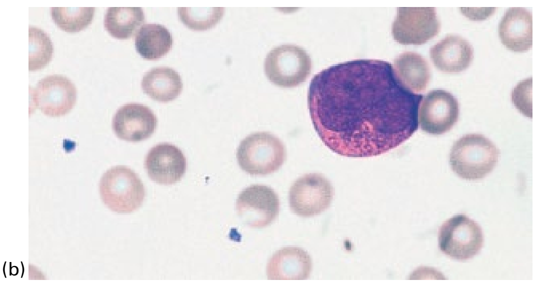


Fig. 1.2 PB film of a patient with AML showing: (a) a type II blast with scanty azurophilic granules; (b) a promyelocyte with more numerous granules and a Golgi zone in the indentation of the nucleus. MGG × 100.

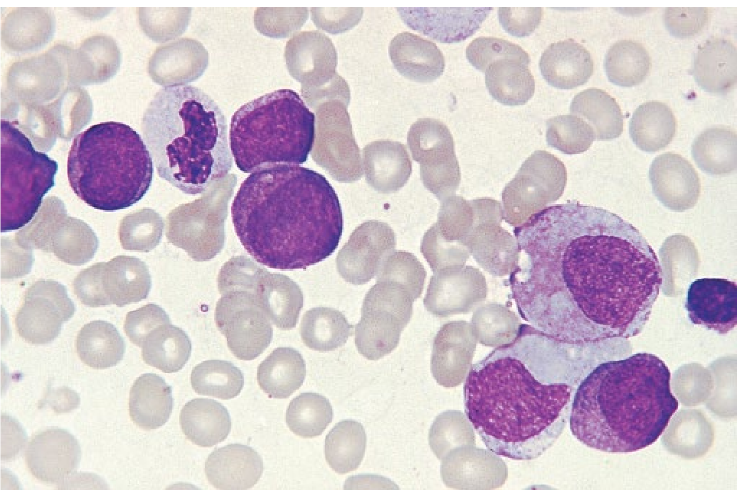
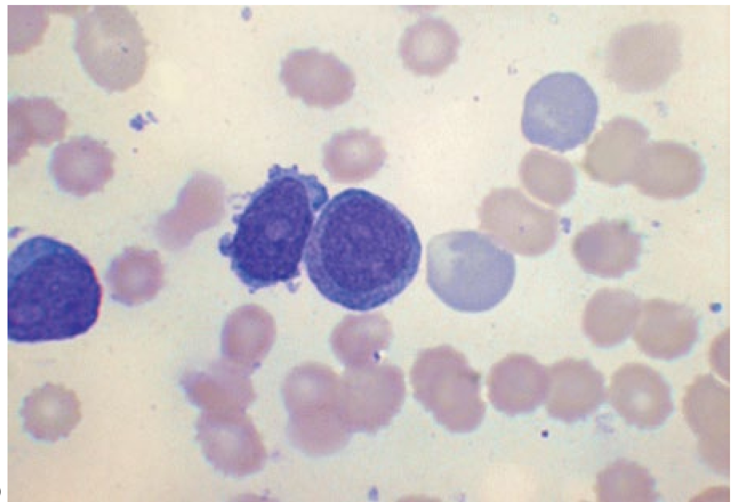
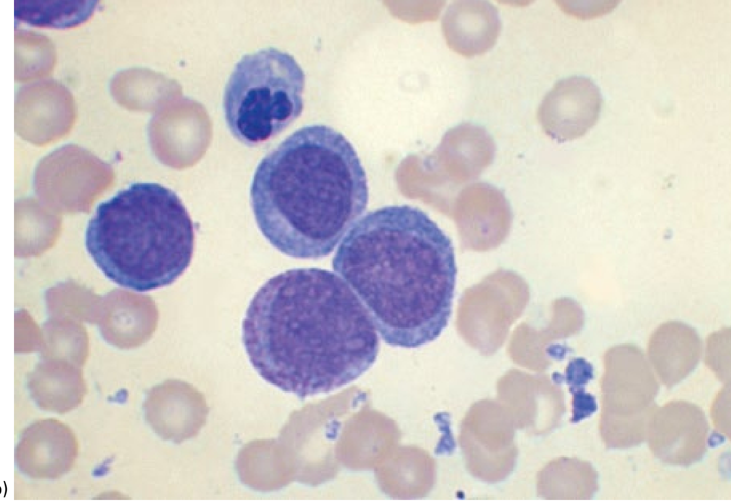


Fig. 1.3 Bone marrow (BM) film of a patient with AML – French–American – British (FAB) M2/t(8;21)(q22;q21.2) – showing a cell that has scanty granules but nevertheless would be classified as a promyelocyte rather than a blast because of its low nucleocytoplasmic ratio; defective granulation of a myelocyte and a neutrophil is also apparent. Type I and type II blasts are also present. MGG × 100.

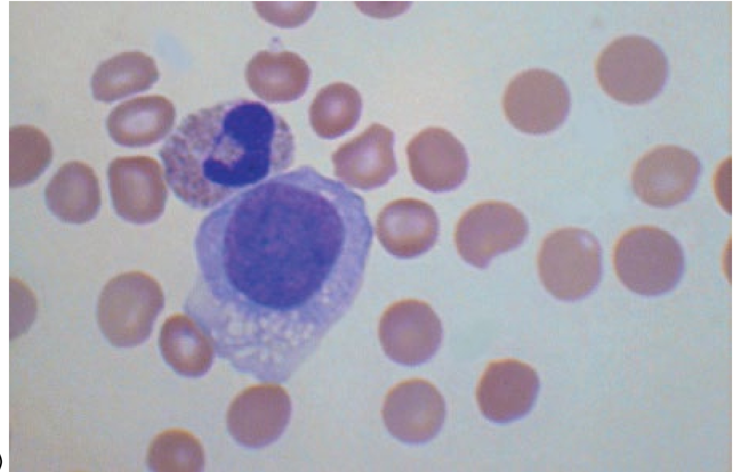


(a)

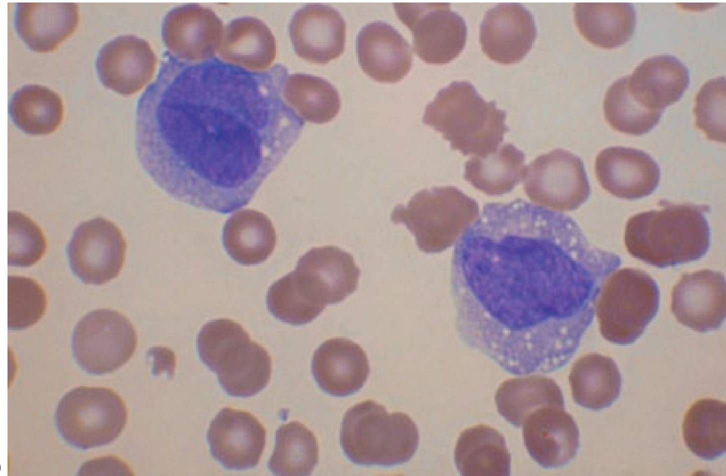


(b)

Fig. 1.4 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.



(a)



(b)

Fig. 1.5 BM film from a patient with FAB type M5 AML showing: (a) a monoblast and a neutrophil; (b) two promonocytes. MGG × 100.

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.

A 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 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 1.5b and 1.6) 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.

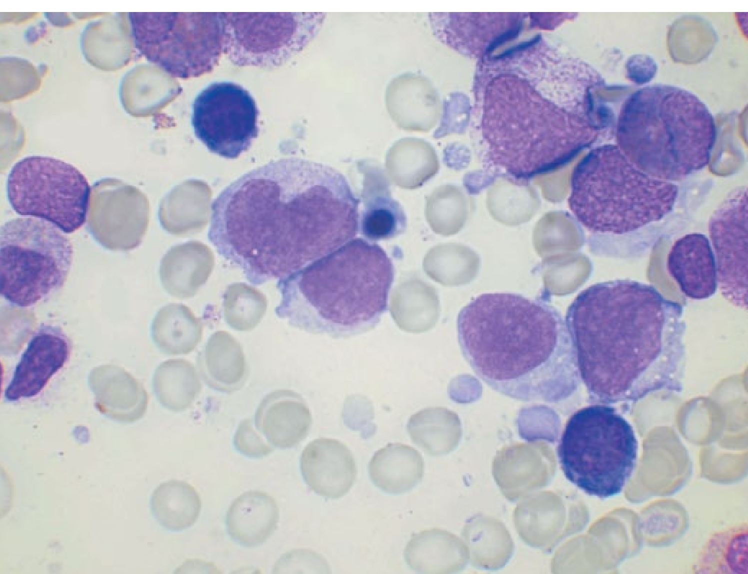


Fig. 1.6 BM film from a patient with FAB type M5 AML showing a promonocyte and three monoblasts; the promonocyte has an irregular nucleus but otherwise is very similar to the three monoblasts. MGG × 100.

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 [5–9]. It deals with both diagnosis and classification.

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 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 are 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 23) are present.

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 136).

†It should be noted that in the 2016 revision of the 2008 WHO classification such cases are no longer recognized as AML unless 20% of all cells are blast cells [10].

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 [9]. It should be noted that SBB is more sensitive than MPO in the detection

‡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.

of myeloid differentiation, and more cases will be categorized as M1 rather than M0 if it is used [20].

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 [21]. Cytochemical demonstration of MPO activity can give prognostic information, since a higher percentage of MPO-positive blasts is strongly associated with a better prognosis [22]. 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 [23]. A bone marrow blast percentage of less than 5% has been validated as a criterion [24]. 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 [23]. Similarly, a molecular complete remission requires that there be no molecular evidence of minimal residual disease [23].

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 maturation (M1) and with granulocytic maturation (M2), 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 200) 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 page 54) 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 [25–30] (Table 1.3).
2) The demonstration of cytoplasmic MPO activity by ultrastructural cytochemistry [26,31,32] (Table 1.4; Fig. 1.9).
3) The demonstration of cytoplasmic MPO protein by immunocytochemistry or flow cytometric immunophenotyping 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 [33] but its expression may not be restricted to myeloid cells [34].

Flow cytometric immunophenotyping is now widely used for identifying cases of M0 AML and as a consequence other techniques are now largely redundant. However, alternative 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 [35].

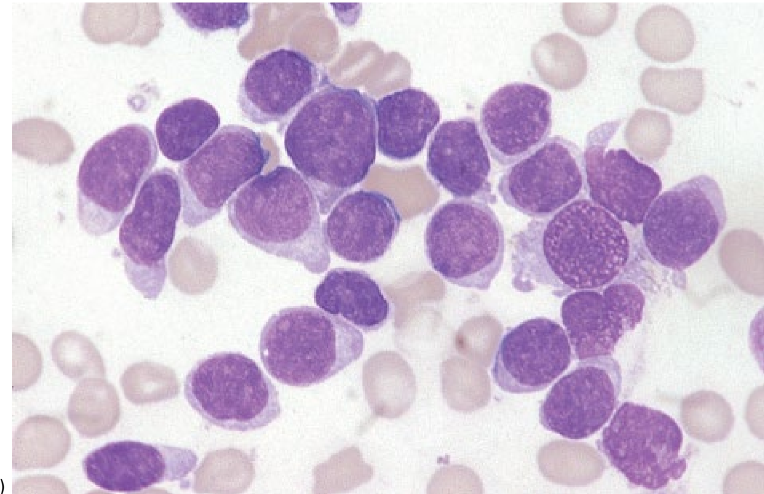
M0 AML has been associated with older age, higher WBC, adverse cytogenetic abnormalities and poor prognosis [35–37]. The molecular genetic abnormalities recognized include a high incidence of loss-of-function mutations of the *RUNX1* gene, most of which are biallelic [35,38]. In a study of 20 genes in 67 patients with leukaemia

*CD = Cluster of Differentiation.

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.


(a)

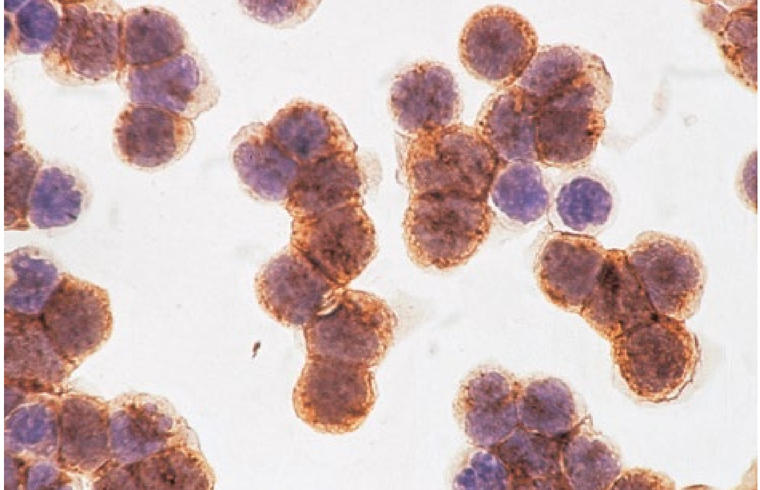

(b)

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, human leucocyte antigen (HLA)-DR and terminal deoxynucleotidyl transferase (TdT). Immunoperoxidase × 100.

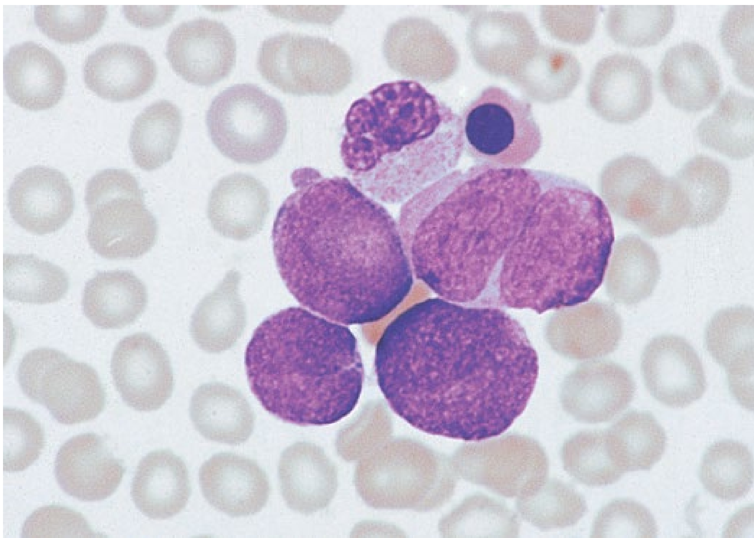


Fig. 1.8 BM film of a patient with FAB M0 AML showing agranular pleomorphic blasts with a high nucleocytoplasmic ratio; the presence of a neutrophil with a nucleus of abnormal shape suggests the correct diagnosis. MGG × 100.

Table 1.3 Ultrastructural characteristics distinguishing blast cells and other immature leukaemic cells from each other [25,26].

Myeloblasts of neutrophil lineage
Small, medium or large granules; sometimes Auer rods, which may be homogeneous or composed of longitudinal tubules or dense material with a periodic substructure [27]
Promyelocytes of promyelocytic leukaemia
In hypergranular promyelocytic leukaemia the cytoplasm is packed with granules ranging from 120 to 1000 nm in diameter [28,29]; 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 others 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 [29]; microfibrils and stellate configurations of rough endoplasmic reticulum are also characteristic of M3 AML, particularly M3 variant [30]
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 [27]
*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 [26]. 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 20)
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 [30]; 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 [30]
Monoblasts and promonocytes
The first granule to appear in a monoblast is a small, peripheral acid phosphatase-positive granule [31]. 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 [26,32]
Proerythroblasts
PPO-like activity may be present in the Golgi zone

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

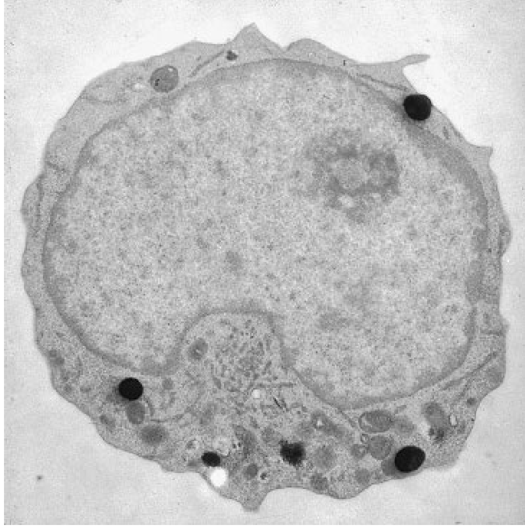


Fig. 1.9 Ultrastructural cytochemistry showing peroxidase-positive granules in a myeloblast. (With thanks to Professor Daniel Catovsky, London.)

defined according to FAB criteria, the genes most often found to be mutated were *FLT3* (28.4%), followed by mutations in *IDH1* or *IDH2* (28.8%), *RUNX1* (23.9%), *NRAS* or *KRAS* (12.3%), *TET2* (8.2%), *DNMT3A* (8.1%), *KMT2A* (7.8%) and *ASXL1* (6.3%) [39].The gene expression profile of M0 AML is distinctive and differs between cases with and without *RUNX1* mutation; the latter show upregulation of B-lineage related genes [40]. 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 [41].

Cytochemical reactions in M0 acute myeloid leukaemia

By definition fewer than 3% of blasts are positive for MPO, SBB and naphthol AS-D chloroacetate esterase (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 [42].

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 [43–45]. 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 [46], 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 [47]. 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 54).

M1 is arbitrarily separated from M2 AML by the requirement that no more than 10% of non-erythroid cells in the bone marrow belong to the maturing granulocytic component (promyelocytes to neutrophils).

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

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.

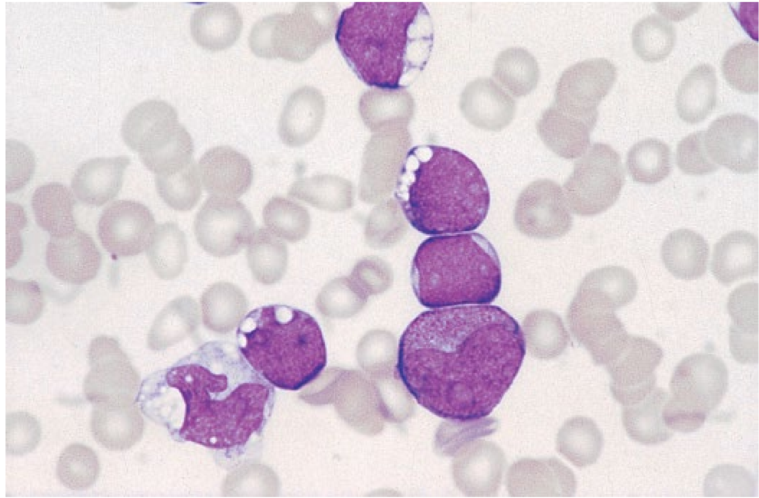


Fig. 1.10 PB film of a patient with FAB M1 AML showing type I and type II blasts, some of which are heavily vacuolated, and a promyelocyte. MGG × 100.

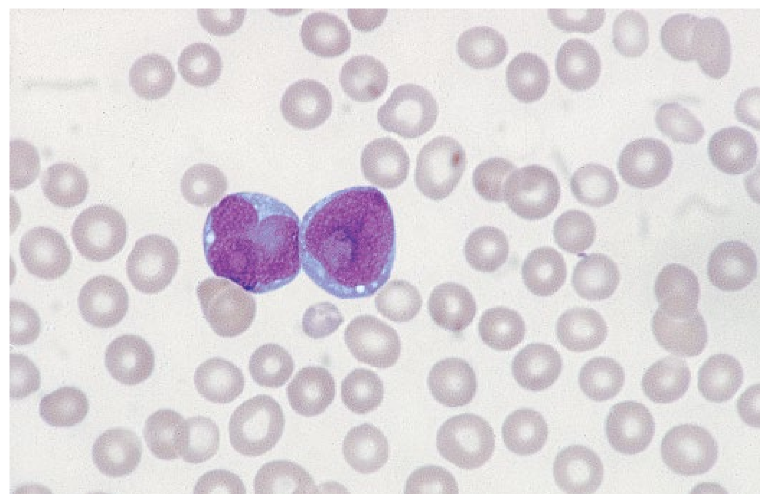


Fig. 1.11 PB film of a patient with FAB M1 AML showing type I blasts with cytoplasmic vacuolation and nuclear lobulation. MGG × 100.

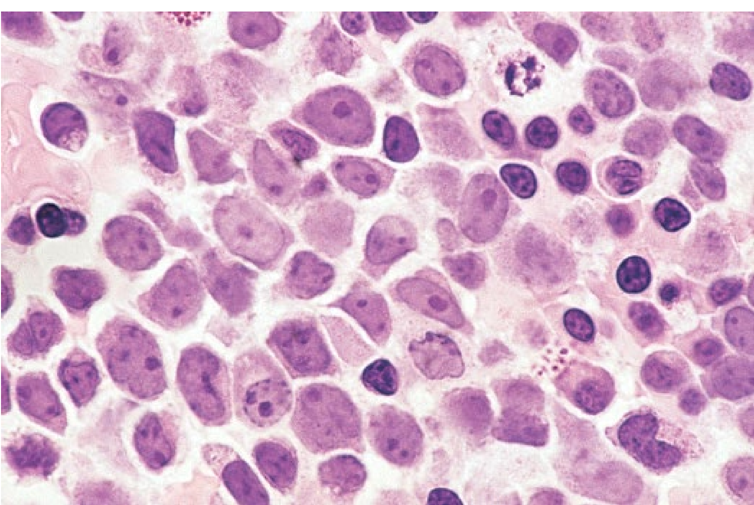
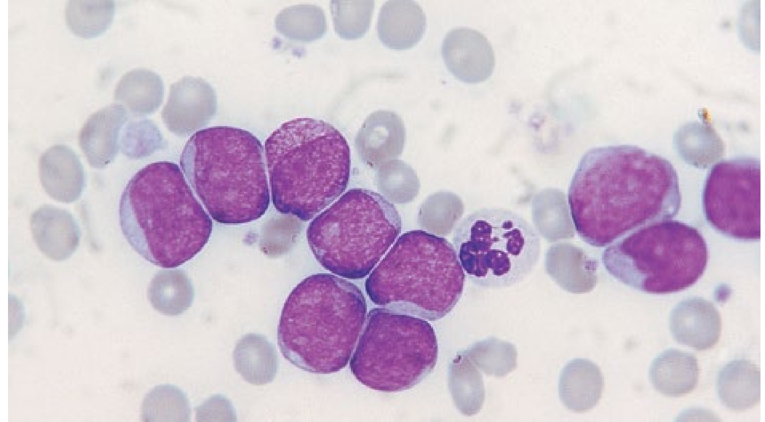
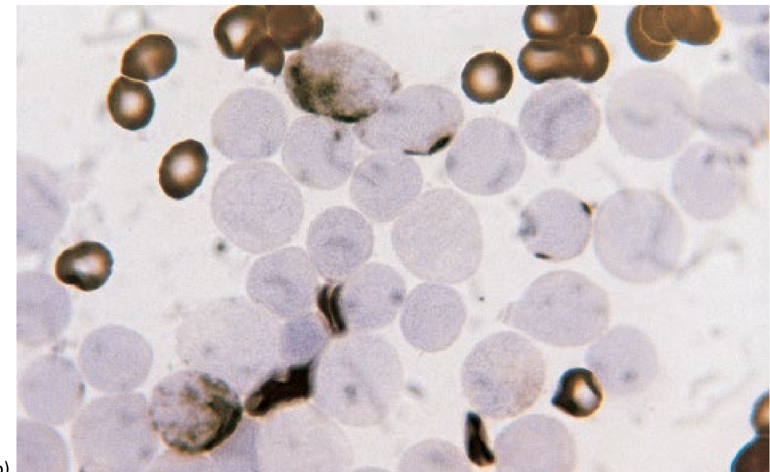


Fig. 1.12 Trephine biopsy section from a patient with FAB M1 AML. The majority of cells present are blasts with a high nucleocytoplasmic ratio and prominent nucleoli; there are also some erythroblasts. Resin embedded, haematoxylin and eosin (H&E) × 100.

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 [12] 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 case 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.


(a)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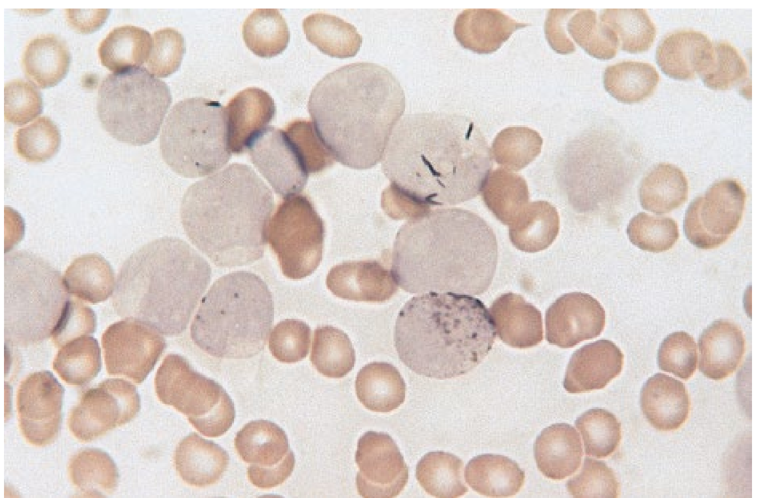

(c)

Fig. 1.13 Cytochemical reactions in a patient with FAB 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.

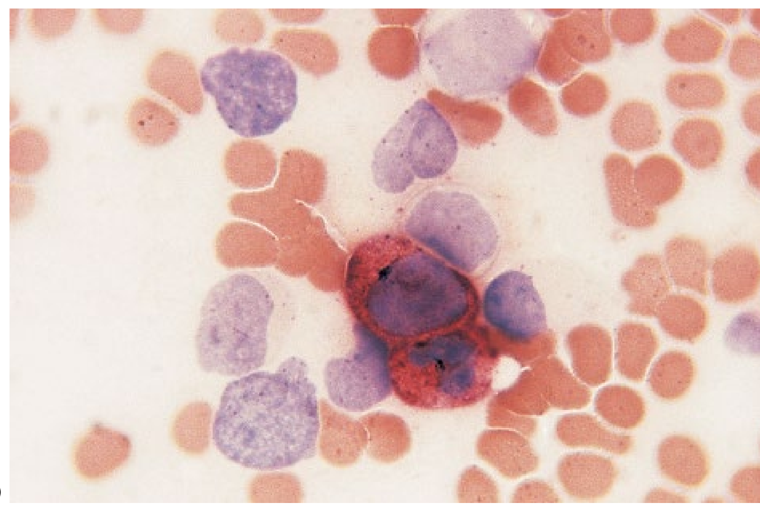


Fig. 1.13 (Continued)
(d) Chloroacetate esterase (CAE) stain of a BM film showing a positive neutrophil and a positive blast; other blasts present are negative. CAE × 100.

Auer rods give positive MPO and SBB reactions and occasionally weak PAS reactions. The reaction for CAE is usually weak or negative [12]. 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

* Granulocytic maturation is intended.

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 [47], probably because of an association between Auer rods and t(8;21) (see page 138). 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

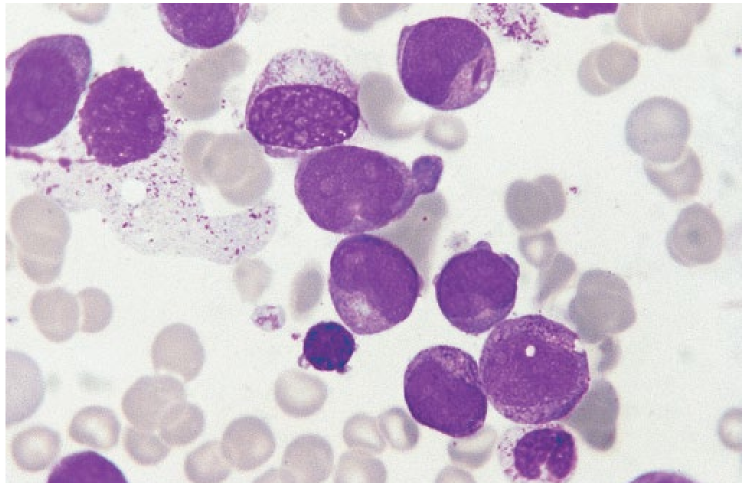
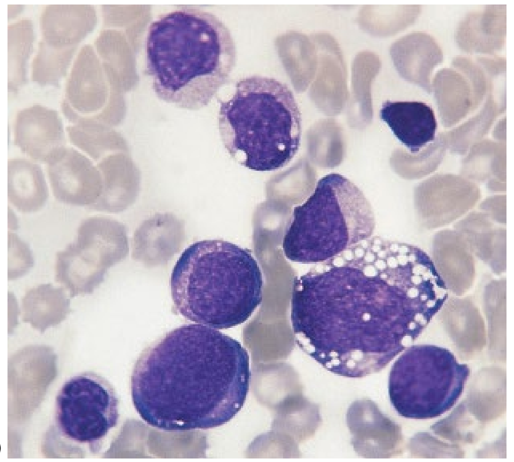


Fig. 1.14 BM film of a patient with FAB M2 AML showing blasts (one of which contains an Auer rod), promyelocytes and a neutrophil. Note the very variable granulation. MGG × 100.



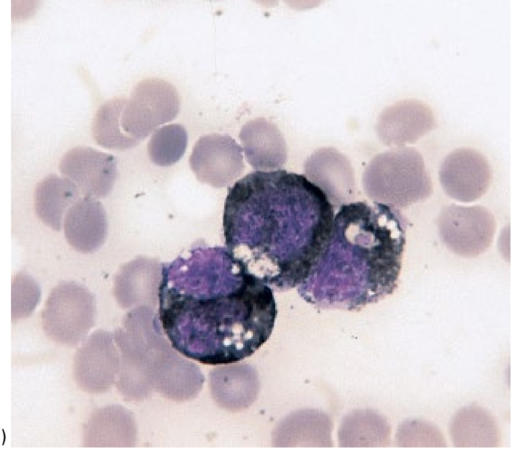


Fig. 1.15 BM film of a patient with FAB M2 AML stained by (a) MGG and (b) SBB. In this patient both blasts and maturing cells were heavily vacuolated. ×100.

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.

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. The reaction for CAE is usually weak or negative [12] except in M2 AML associated with t(8;21) (see page 138) in which Auer rods are often positive for CAE [11]. 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

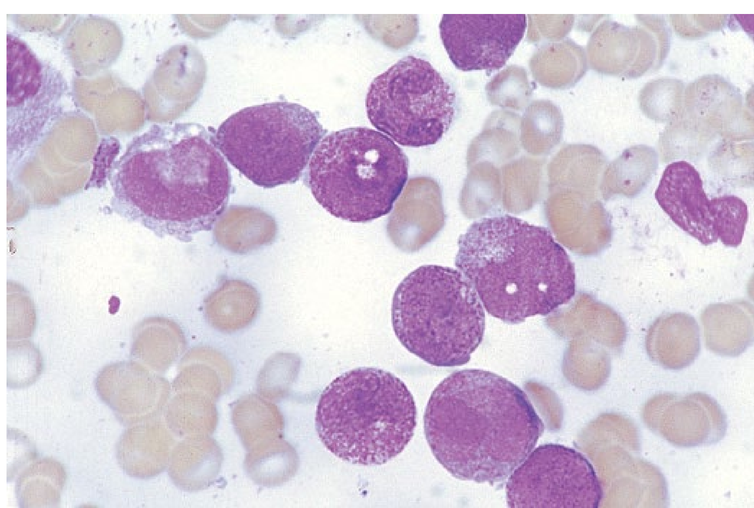


Fig. 1.16 BM film of a patient with FAB M2 AML showing unusually heavy granulation of neutrophils and precursors. MGG × 100. (With thanks to the late Dr David Swirsky.)

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)(q24.1;q21.2) (see page 147), and with abnormal coagulation. There is disseminated intravascular coagulation and activation of fibrinolysis, resulting in abnormal bleeding and bruising (Fig 1.17). This diagnosis can sometimes be suspected from the prominent haemorrhagic manifestations. However, there can also be venous thromboembolism including presentation with pulmonary embolism [48]. Typical cytological and histological features are shown in Figs 1.18–1.20. 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. 1.20). Auer rods are common. In one series they were noted in fewer than 50% of cases [49], but others have observed them to be almost always present, at least in a minority of cells [50]. In some cases there are giant granules or multiple Auer rods, which are often present in sheaves or 'faggots' (Fig. 1.19). Bundles of Auer rods are uncommon in other types of AML but are occasionally seen, reported, for example, in a patient with acute myelomonocytic leukaemia with eosinophilia associated with inv(16) [51] and in a patient with del(5q) without rearrangement of *RARA* [52]. 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

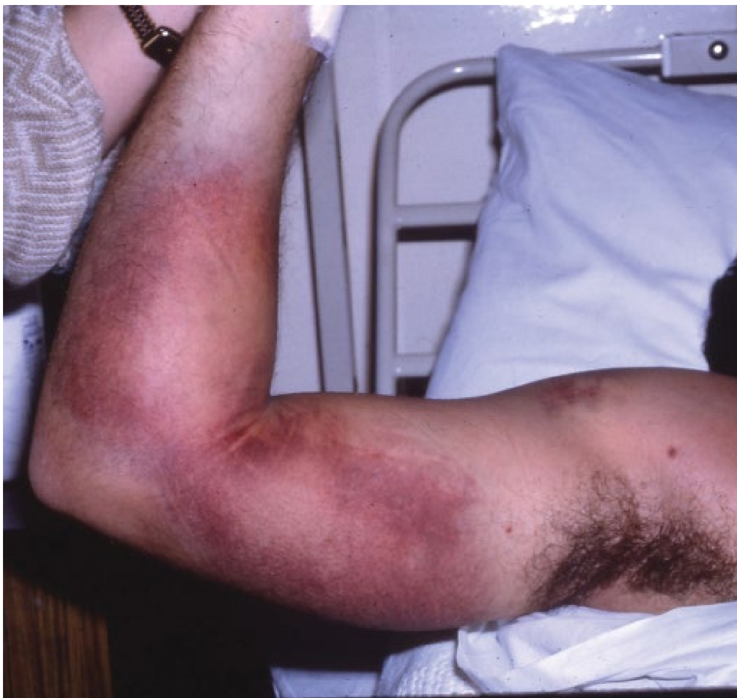


Fig. 1.17 Clinical photograph of a patient with FAB M3 AML showing extensive spontaneous bruising of the arm.

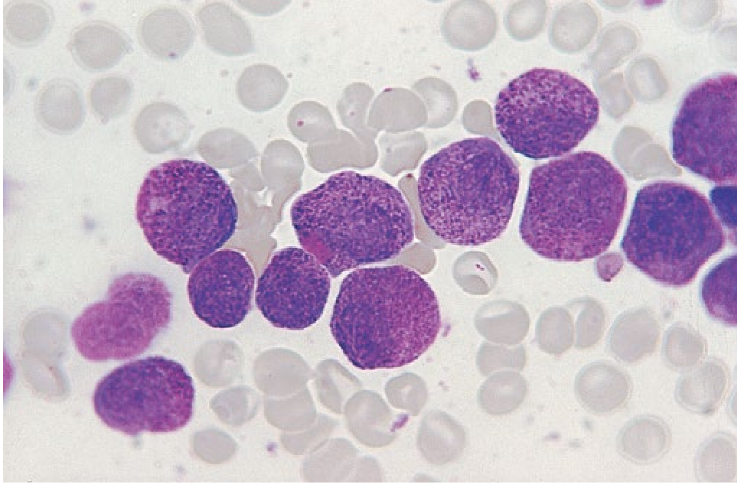


Fig. 1.18 BM film of a patient with FAB M3 AML showing hypergranular promyelocytes, one of which has a giant granule. MGG × 100.

pink material in the cytoplasm may also be seen. There may be basophilic differentiation in M3 AML, in addition to the dominant neutrophilic differentiation. Bone marrow macrophages may contain giant granules or Auer rods derived from ingested leukaemic cells (Fig. 1.21). Auer rods can persist in macrophages after the patient has entered complete remission [53]. 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

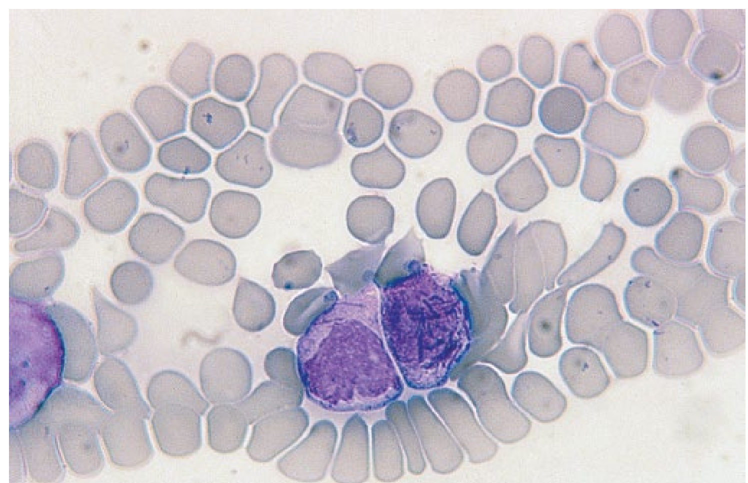


Fig. 1.19 PB film of a patient with FAB M3 AML. One of the abnormal promyelocytes contains loose bundles of Auer rods. MGG × 100.

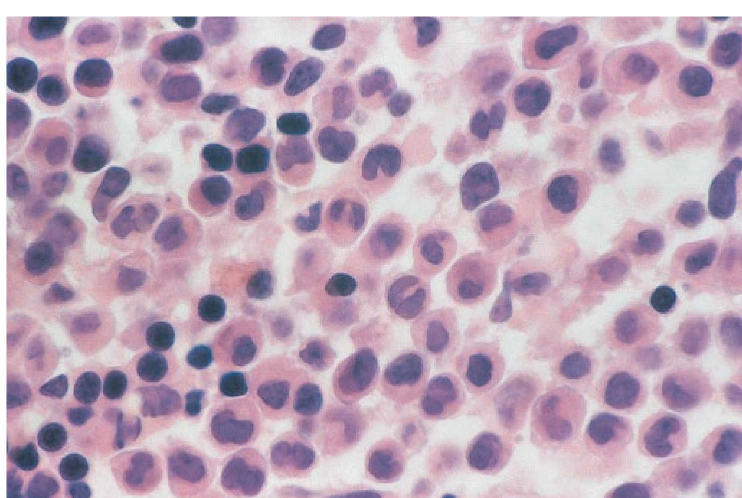


Fig. 1.20 Trephine biopsy section from a patient with FAB M3 AML. Paraffin embedded, H&E × 100.

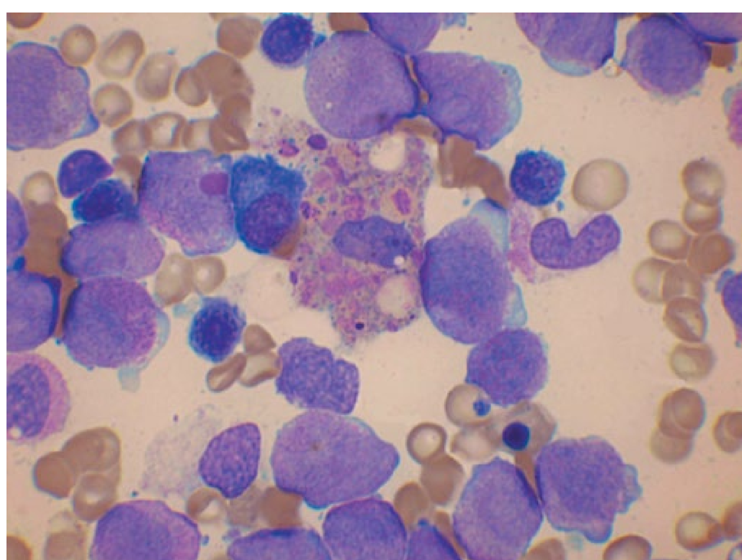


Fig. 1.21 BM film from a patient with acute promyelocytic leukaemia a few days after starting treatment with all-*trans*-retinoic acid (ATRA): one leukaemic cell contains a giant granule; a prominent macrophage contains granules and Auer rods from ingested leukaemic cells. MGG × 100.

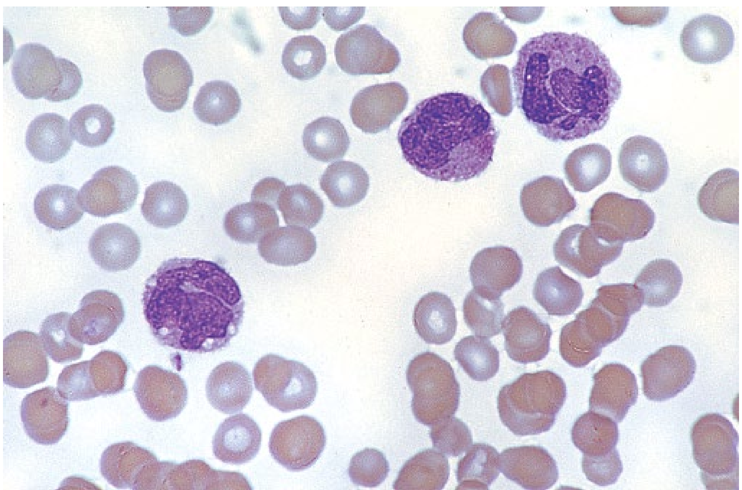


Fig. 1.22 PB film of a patient with FAB M3 AML being treated with ATRA and granulocyte colony-stimulating factor (G-CSF); leukaemic promyelocytes are undergoing maturation into highly abnormal cells. MGG × 100.

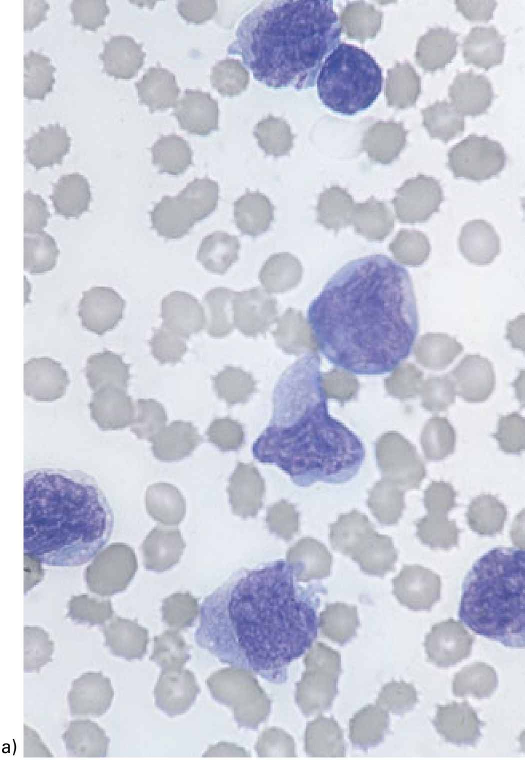
is often low and, even when there is a leucocytosis, typical hypergranular promyelocytes may not be present in the blood. The specimen may clot during attempted aspiration, as a consequence of the associated hypercoagulable state, but usually sufficient cells are obtained for diagnosis.

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. 1.22). Metamyelocytes and neutrophils may contain Auer rods [54]. The neutrophil count rises and in some patients also the basophil count [55]. Following treatment with ATRA, the terminally differentiated cells in a late stage of apoptosis are engulfed by bone marrow macrophages [56]. M3 AML is also responsive to treatment with arsenic trioxide, As_2O_3. Hyperleucocytosis may occur during therapy with both ATRA and arsenic trioxide [57].

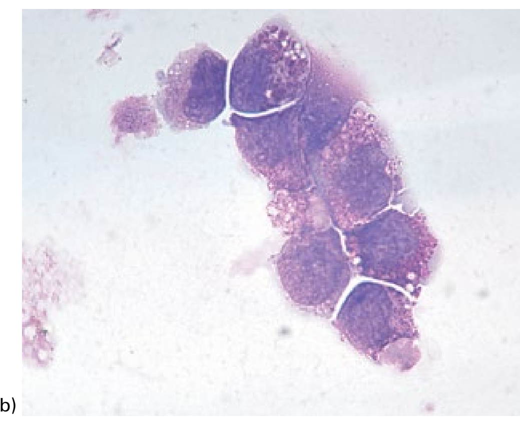
The variant form of acute promyelocytic leukaemia: M3 variant acute myeloid leukaemia

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 [58–60]. 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 [60] (Fig. 1.23) and, conversely, cases of M3 AML may have less granular cells on relapse [29]. 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. 1.24). A variable proportion of cells may have multiple Auer rods, fine dust-like granules, or large oval, elliptiform or somewhat angular cytoplasmic inclusions with the same staining characteristics as primary granules. Typical hypergranular promyelocytes constitute a small minority of the leukaemic cells in the peripheral



(a)



(b)

Fig. 1.23 (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. (With thanks to the late Professor David Grimwade.)

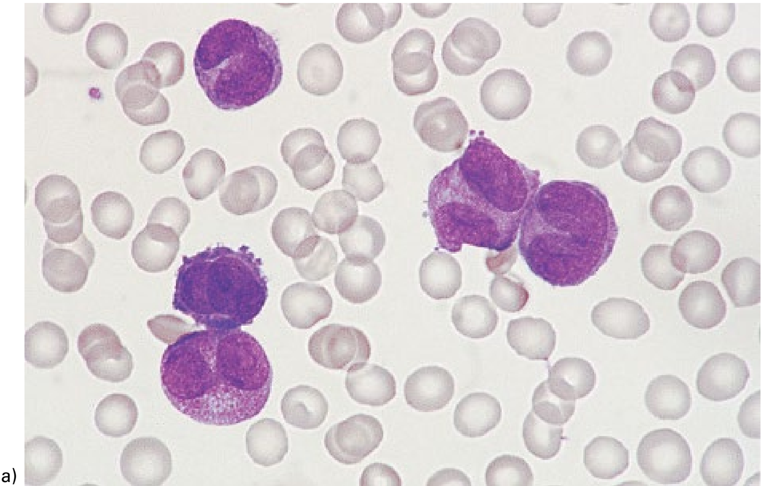
blood but they are usually more numerous in the bone marrow. On ultrastructural examination, the granules are smaller and usually less numerous than in hypergranular promyelocytic leukaemia [29] (Fig. 1.25). The WBC is usually higher in M3V than in M3 AML.

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 megakaryoblasts [29] (Fig. 1.26). Hypergranular promyelocytes and Auer rods may be totally absent [61]. However, it is important to examine crushed cells in blood films as Auer rods may then be apparent [62]. Hyperbasophilic promyelocytes 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 (Fig. 1.25) [29]. Occasional cases have a hand-mirror conformation [63].

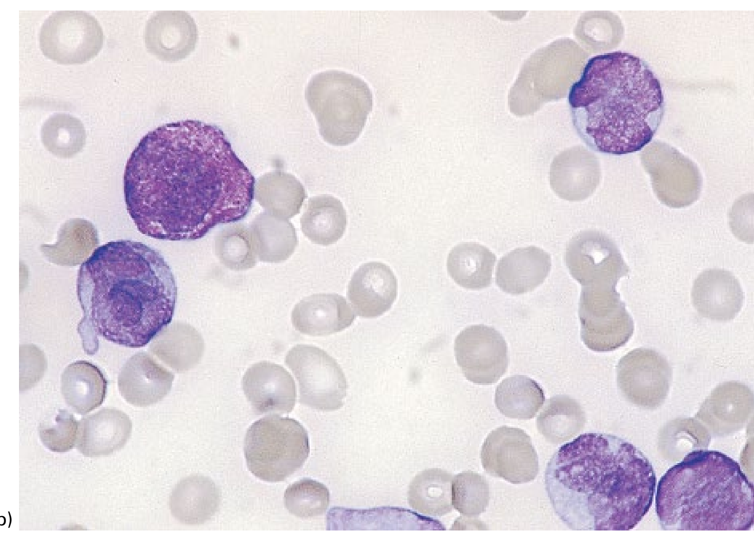
M3V may be confused with acute monocytic leukaemia (M5b) if blood and bone marrow cells are not examined carefully and if the diagnosis is not considered. The use of an automated blood cell counter based on cytochemistry (MPO or SBB) is useful for the rapid distinction between M3V and M5 AML (see Fig. 1.70). 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 alone, the prognosis of M3 variant was somewhat worse than that of M3 AML [64]. This is likely to be related to the higher WBC, since the WBC is of prognostic importance in M3/M3V AML [37], and the greater prevalence of a secondary mutation, an internal tandem duplication 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 [64] 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.



(a)



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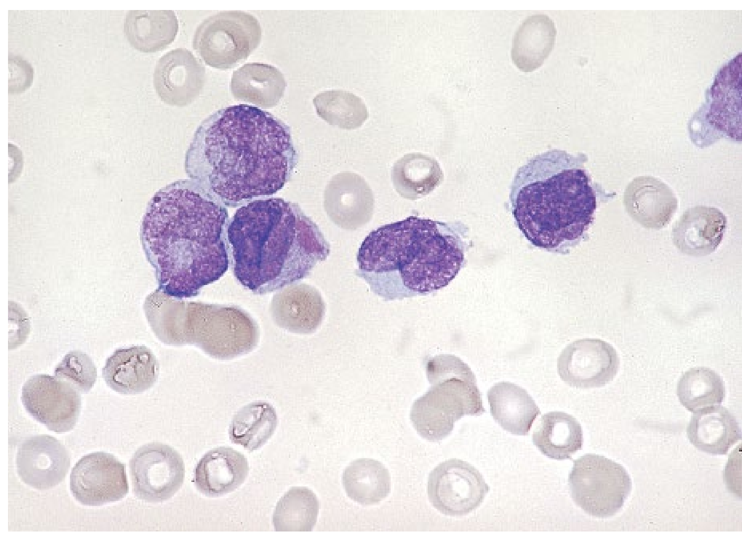
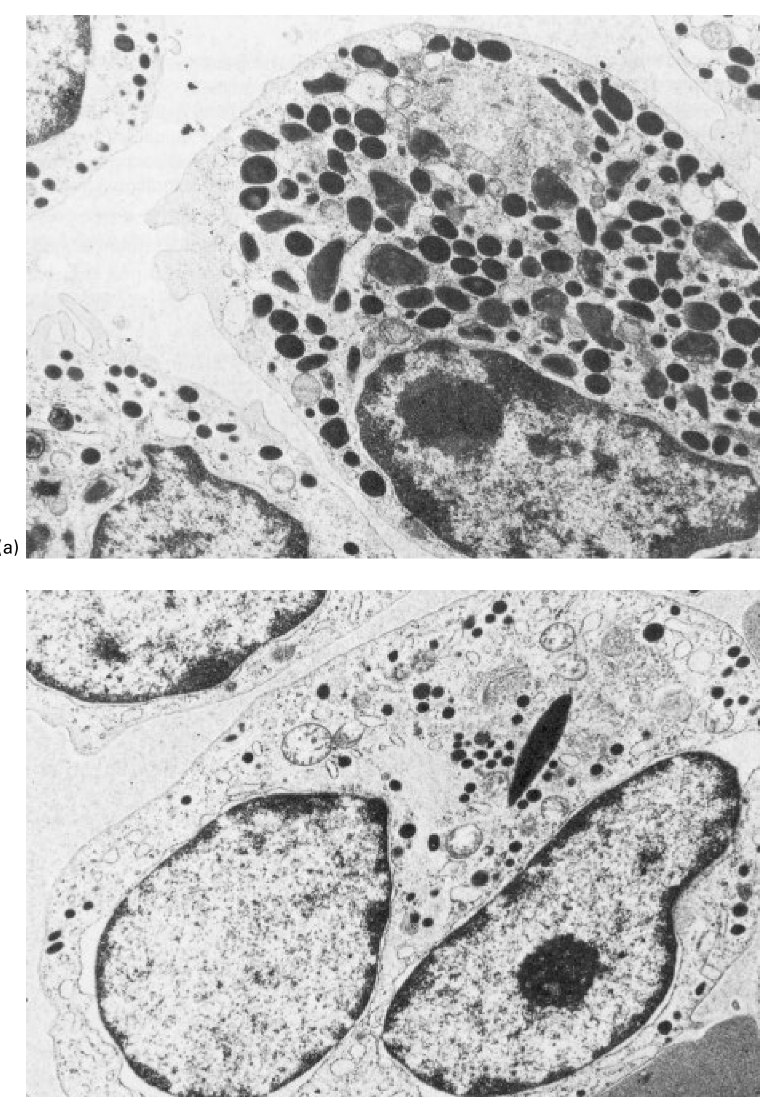


(c)

Fig. 1.24 (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. (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.



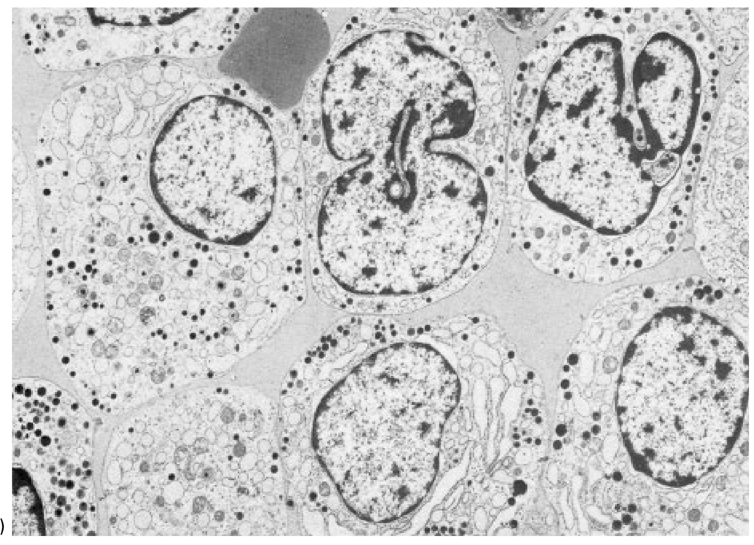


Fig. 1.25 Ultrastructural examination of leukaemic cells of three patients with acute promyelocytic leukaemia: (a) typical M3 AML showing granules that are numerous and large; (b) M3 variant AML showing a lobulated nucleus, granules that are smaller and more sparse, and one Auer rod; (c) hyperbasophilic M3 variant AML showing fewer and smaller granules than in typical M3 AML plus abundant dilated rough endoplasmic reticulum. Uranyl acetate, lead citrate stain. (With thanks to Dr Robert McKenna, Minnesota, and by permission of the *British Journal of Haematology*.)

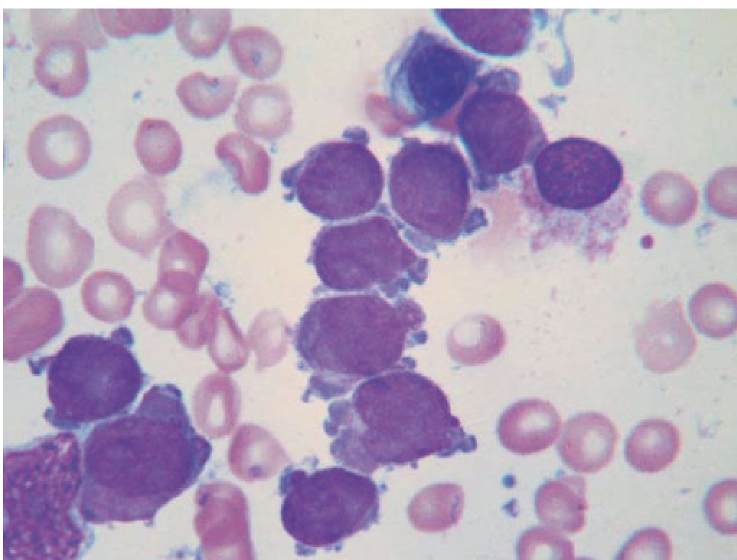


Fig. 1.26 BM film from a patient with the hyperbasophilic variant of acute promyelocytic leukaemia showing abnormal promyelocytes with blebbed basophilic cytoplasm; some also have a dusting of fine cytoplasmic granules. MGG × 100.

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 [65]. 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 [29] (Fig. 1.27) but sometimes the reactions are weaker [66]. A potentially confusing cytochemical reaction in both M3 and M3V AML is the presence in some cases of NSE activity [28,29,49], 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 [66]. 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 [49]. 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 [49].

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 PAS positive [67]. 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 [12].

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 1.28 and 1.29. 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 be at least 20% of

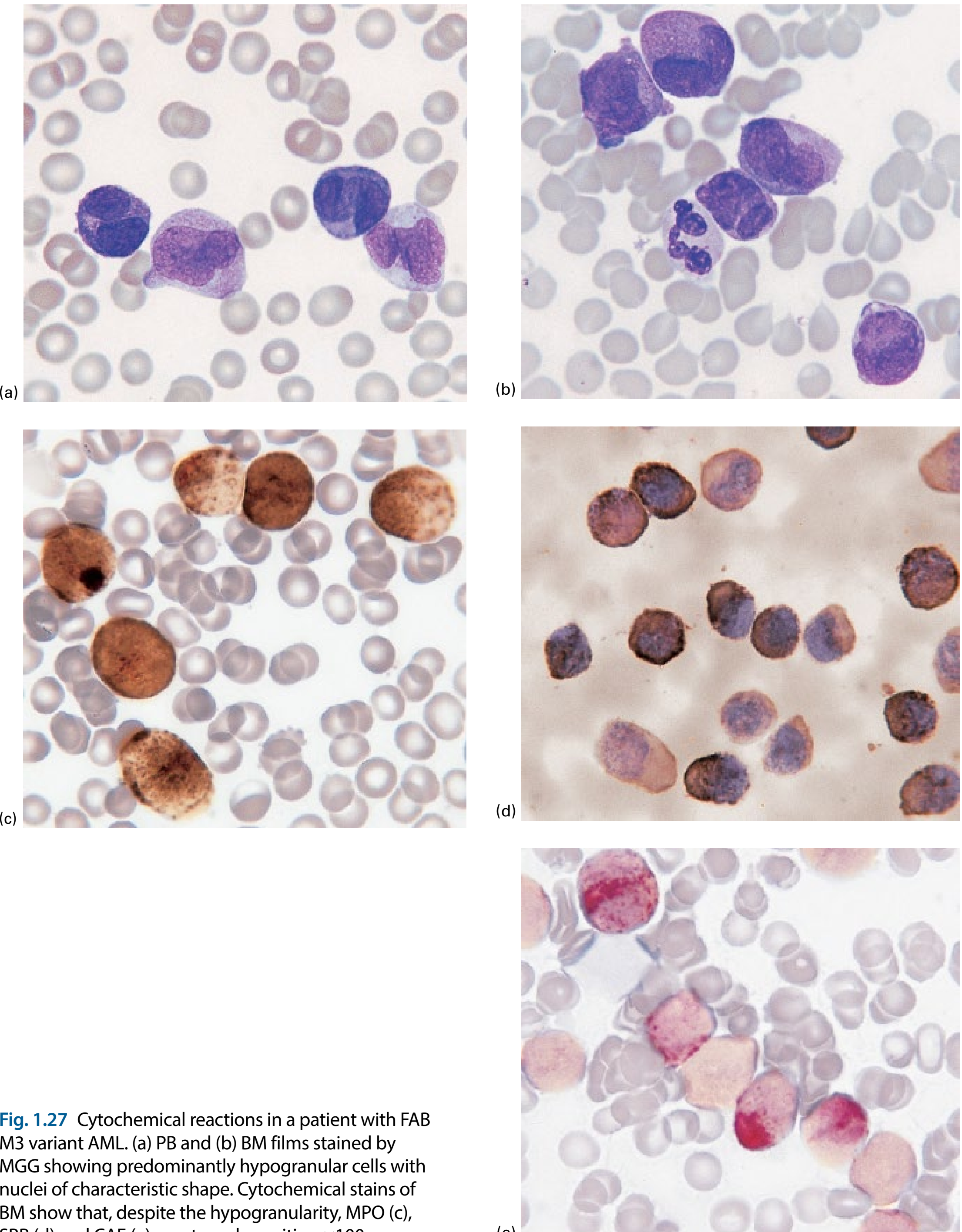
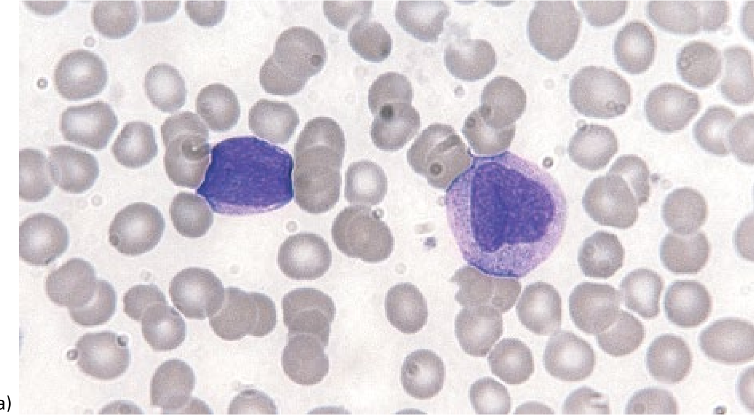


Fig. 1.27 Cytochemical reactions in a patient with FAB M3 variant AML. (a) PB and (b) BM films stained by MGG showing predominantly hypogranular cells with nuclei of characteristic shape. Cytochemical stains of BM show that, despite the hypogranularity, MPO (c), SBB (d) and CAE (e) are strongly positive. ×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 $\geq 5 \times 10^9$/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 $\geq 5 \times 10^9$/l and confirmed by cytochemistry or increased serum or urinary lysozyme concentration

(a)



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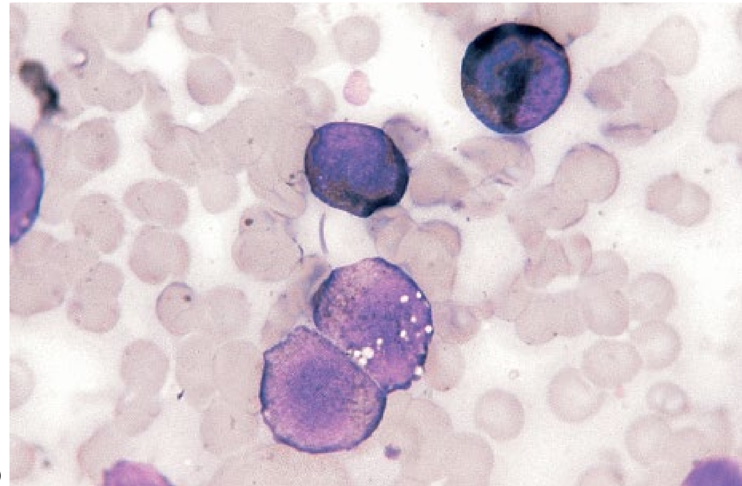


Fig. 1.28 (a) PB film of a patient with FAB M4 AML showing a myeloblast of medium size with a high nucleocytoplasmic ratio and a monoblast that is larger with more plentiful cytoplasm and a folded nucleus with a lacy chromatin pattern. MGG × 100. (b) BM of the same patient stained with SBB showing two monoblasts with a weak granular reaction and two cells of the granulocytic series with a much stronger reaction. SBB × 100.

non-erythroid cells. The recognition of a significant monocytic component requires two criteria to be satisfied, both of which may be 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 [5], or the presence of ANAE activity [8]. 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 [8]. 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. 1.29), basophilic (M4Baso) or both (Fig. 1.30). A rare observation is the presence of grey-green crystals in bone marrow macrophages [68]. When there is eosinophilic differentiation, Charcot–Leyden crystals may be seen.

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

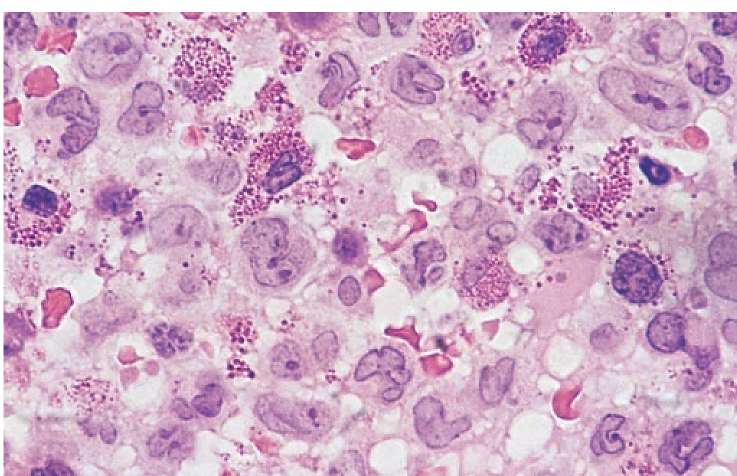
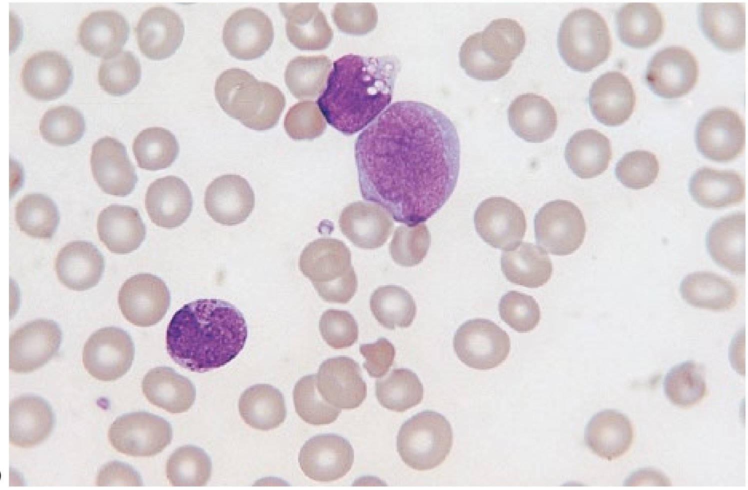
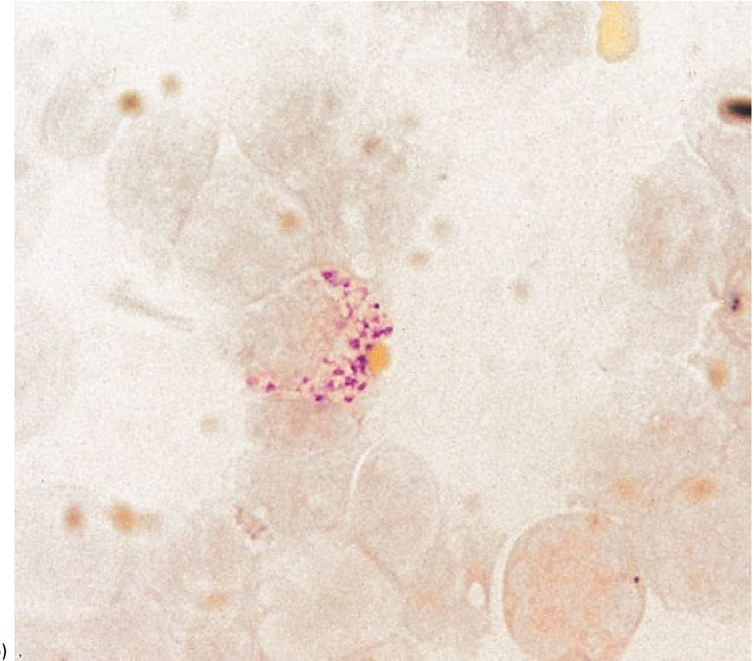


Fig. 1.29 Trephine biopsy section from a patient with M4Eo AML. Cells are either monoblasts/promonocytes, recognized as large cells with round or lobulated nuclei containing prominent nucleoli, or eosinophils. Resin embedded, H&E × 100.



(a)

Fig. 1.30 PB film of a patient with FAB 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)

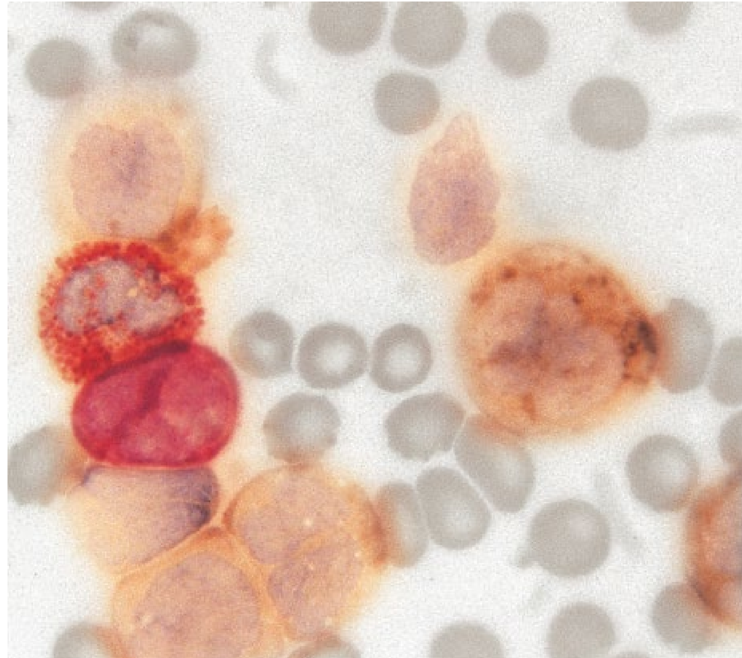

(c)

Fig. 1.30 (Continued)
(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.

(neutrophil lineage) and ANAE (monocyte lineage) [69] is a convenient method for demonstrating the pattern of differentiation and maturation in M4 AML (Fig. 1.30c).

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 1.31–1.34. This diagnosis may be suspected from clinical features when there is infiltration of the skin and the gums (Figs 1.35 and 1.36). Disseminated intravascular coagulation and increased fibrinolysis are more common in M5 AML than in other categories of AML, with the exception of M3 [70]. 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. 1.33). Occasionally leukaemic cells are phagocytic, particularly but not only in patients with t(16;21)(p11.2;q22.2) or with t(8;16)(p11.2;p13.3) or a related abnormality; in one patient with a normal karyotype the clinicopathological features of a haemophagocytic syndrome were present [71]. Monocytic differentiation can be confirmed by cytochemistry and by measurement of urinary and serum lysozyme concentrations; immunophenotyping can also be helpful.

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

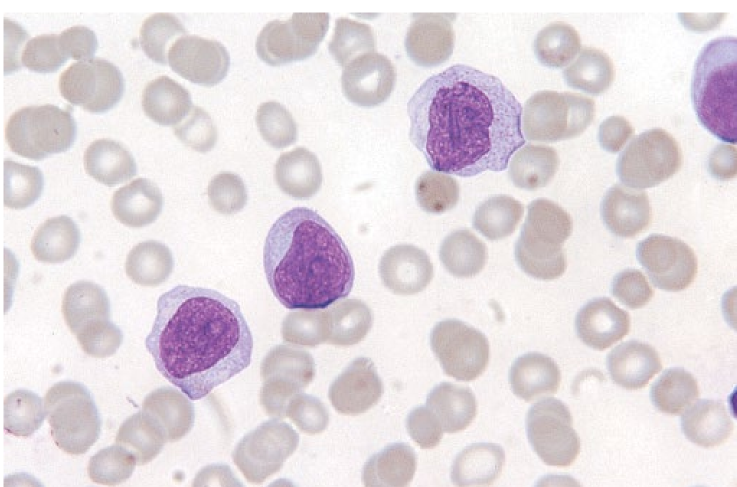


Fig. 1.31 PB film of a patient with FAB M5a AML showing two monoblasts and a promonocyte. MGG × 100.

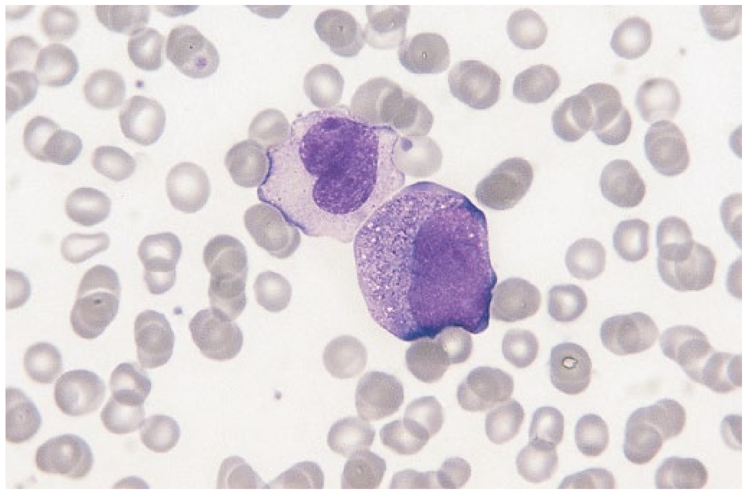
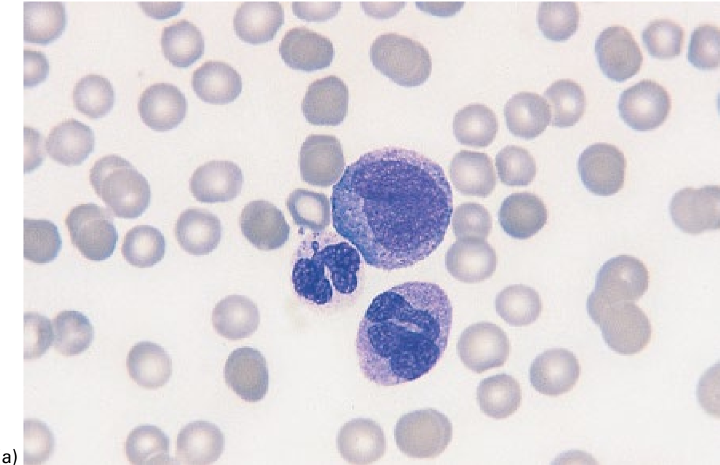
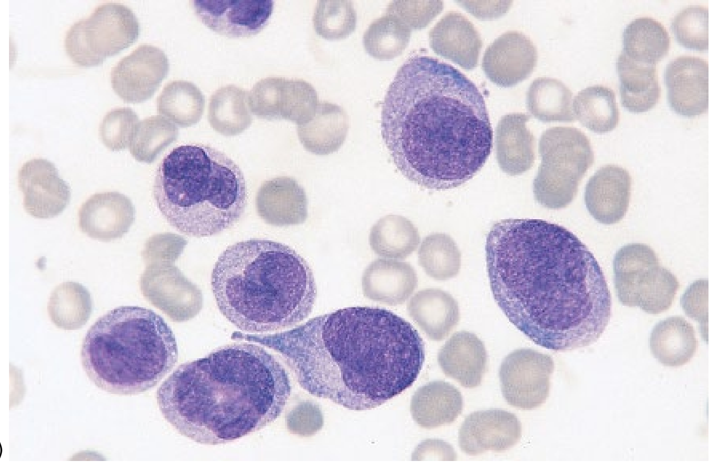


Fig. 1.32 PB film of a patient with FAB M5b AML showing a monocyte and a promonocyte; the latter is moderately heavily granulated. MGG × 100.



(a)



(b)

Fig. 1.33 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.

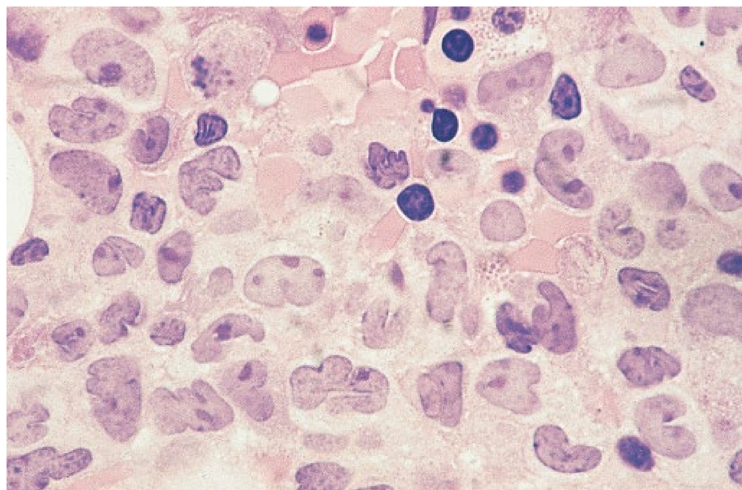


Fig. 1.34 Trephine biopsy section from a patient with FAB M5b AML and myelodysplasia. Monoblasts and monocytes can be identified; the former are the larger cells with a round nucleus, a dispersed chromatin pattern and prominent nucleoli whereas the latter have lobulated nuclei and lack nucleoli. Promonocytes, with intermediate characteristics, are also present. The cells with smaller dark nuclei are erythroblasts, one of which has a nucleus of abnormal shape. Resin embedded, H&E × 100.

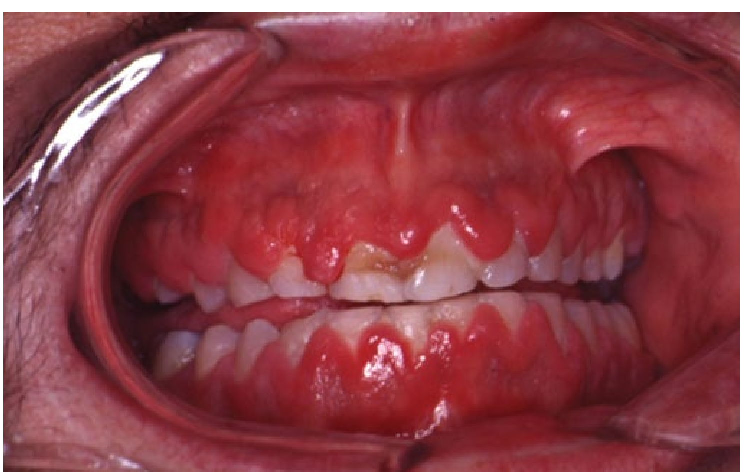


Fig. 1.35 Clinical photograph of a patient with FAB M5a AML showing infiltration of the gums (With thanks to Dr Devinder Gill, Brisbane.)

In a rare form of acute monocytic leukaemia, cells have cytological features resembling those of macrophages or histiocytes (Fig. 1.37). This may be regarded as the leukaemic phase of malignant histiocytosis. The designation M5c has been suggested [72].

M5a and M5b are cytogenetically and genetically distinct. M5a has a significantly higher prevalence of cytogenetic abnormalities with an 11q23 breakpoint and *KMT2A* rearrangement, and also of isolated trisomy 8 [73]; 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 anti-leukaemic drugs, specifically etoposide, cytarabine, anthracyclines, vincristine, asparaginase and cladribine [74].

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 [12] 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

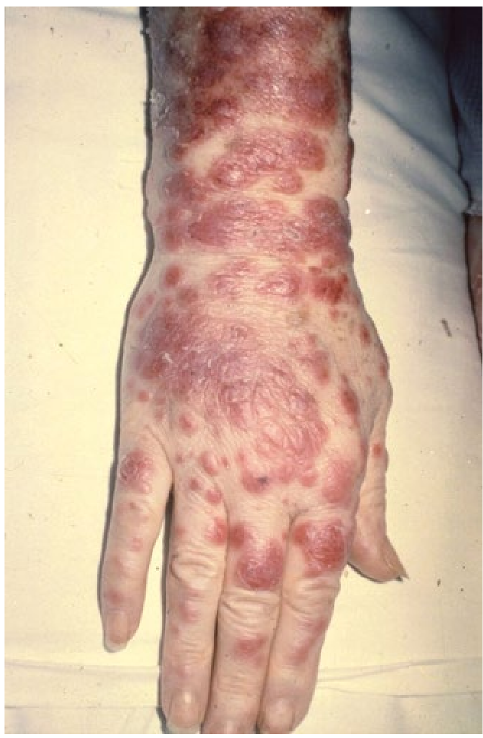


Fig. 1.36 Clinical photograph of a patient with FAB M5b AML showing skin infiltration. (With thanks to Dr Devinder Gill, Brisbane.)

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, that is, for ANAE (Fig. 1.38a), ANBE, NASA (Fig. 1.38b,c) 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, whereas 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 time as MPO activity, can be demonstrated cytochemically (Fig. 1.38d). 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. 1.38e). In M5 AML, the NAP score is usually normal or high in contrast to

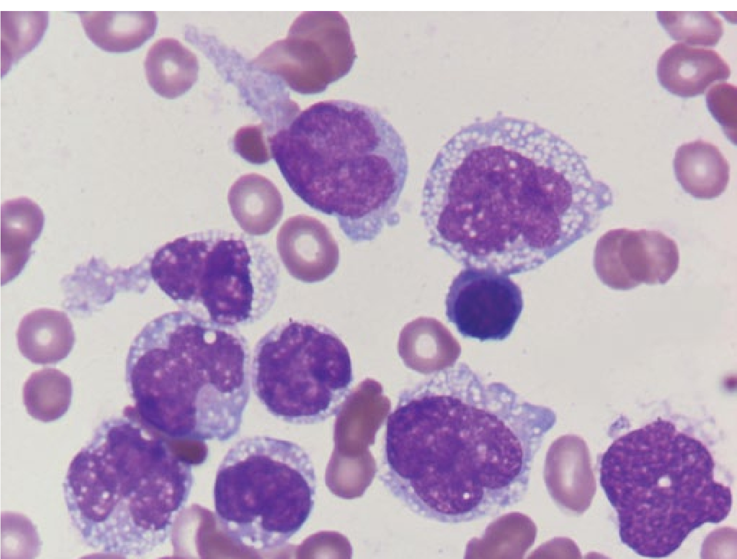


Fig. 1.37 BM film of a patient with leukaemic cells showing histiocytic or macrophage differentiation ('M5c' AML). MGG × 100. (With thanks to Dr Abbas Hashim Abdulsalam.)

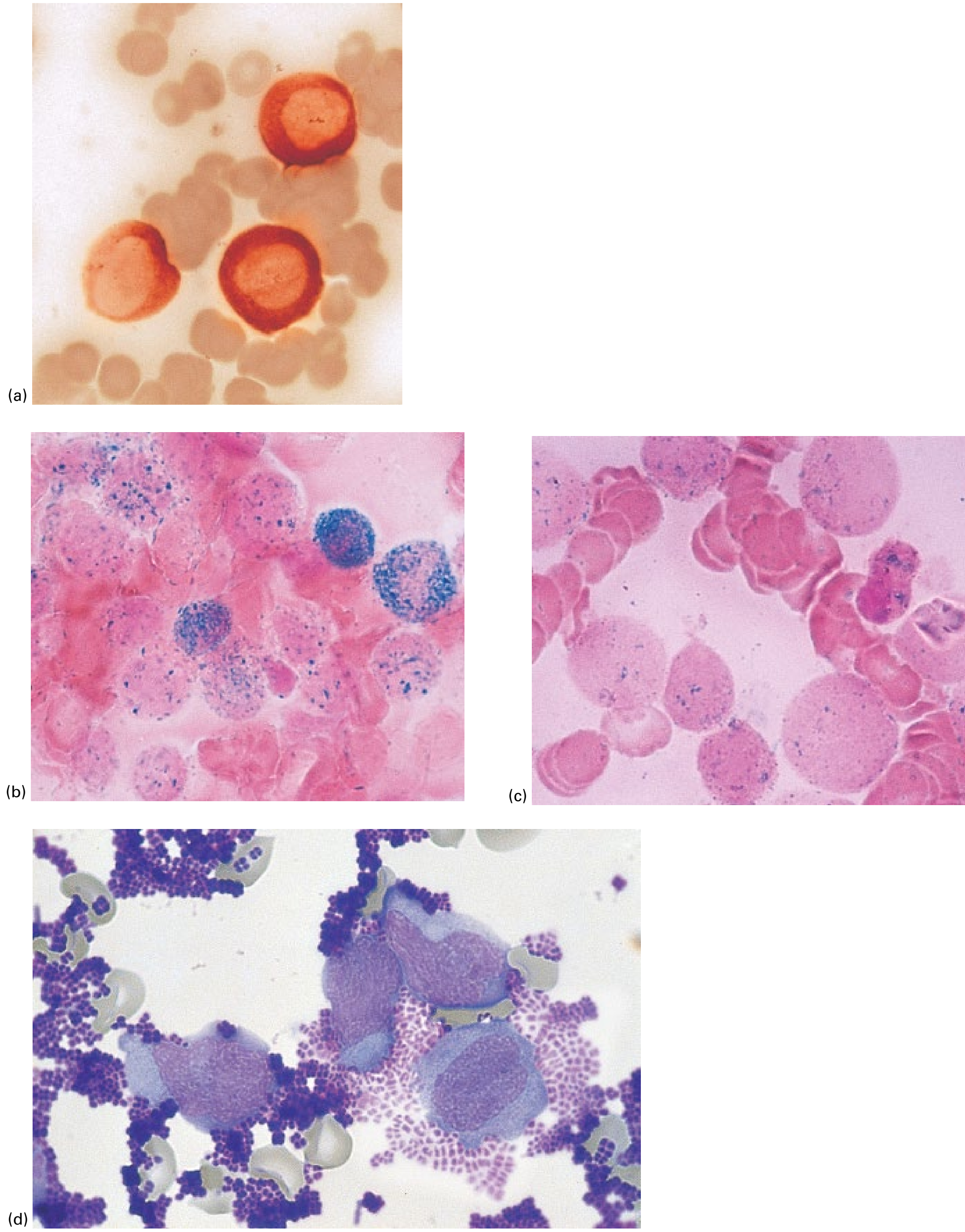
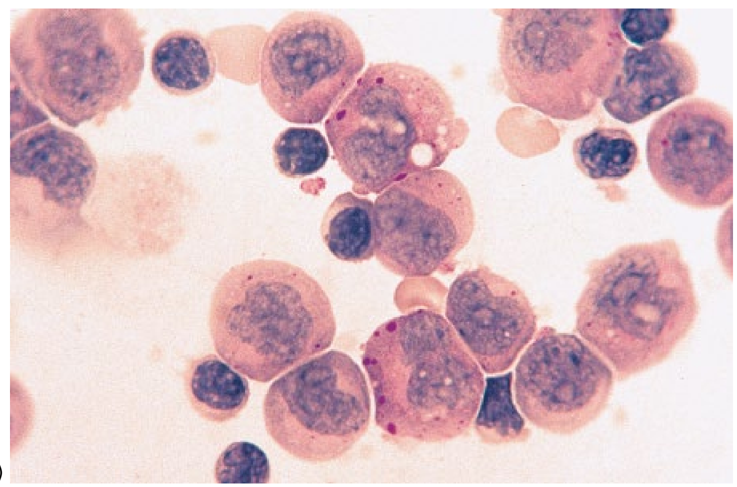


Fig. 1.38 (a) BM film of a patient with FAB M5a AML stained for ANAE activity. ANAE × 100. (b, c) BM film of a patient with FAB M5b AML stained for naphthol AS acetate esterase (NASA) activity without (b) and with (c) fluoride; inhibition of activity by fluoride is apparent. NASA × 100. (d) Lysozyme 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)

Fig. 1.38 (Continued)
(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.

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 would be classified as M0 AML.

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 1.39–1.45. It should be noted that the 2016 WHO definition of erythroleukaemia (see page 194) is very different from the FAB definition. Some cases of M6 AML represent leukaemic transformation of MDS, and a significant proportion of reported cases have been therapy related; however, in one large series only 1 of 62 cases was therapy related with another 2 of 62 being secondary to MDS [75]. Presentation is often with pancytopenia and macrocytosis, with circulating blast cells being present in only a minority of patients [75]. Schistocytes, teardrop cells, pincer cells and basophilic stippling are often present [75]. Circulating erythroblasts are present in around half of patients [75] and may show dysplastic features. Circulating micromegakaryocytes are present in a significant minority [75]. 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. 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

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

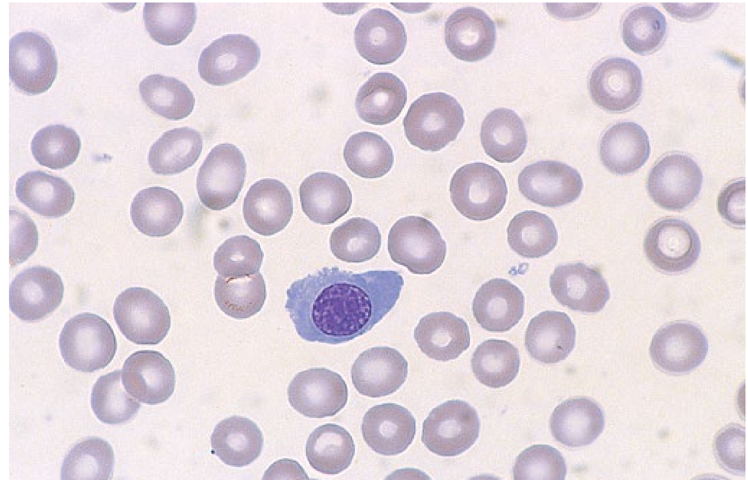


Fig. 1.39 PB film in a patient with FAB M6 AML showing anaemia, severe thrombocytopenia and an abnormal circulating erythroblast. MGG × 100.

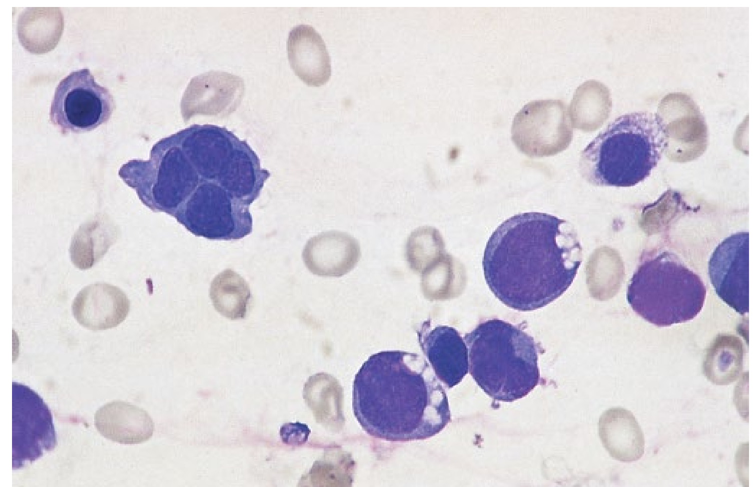


Fig. 1.40 BM film from a patient with FAB M6 AML (erythroleukaemia) showing a multinucleated erythroblast and two heavily vacuolated myeloblasts. MGG × 100.

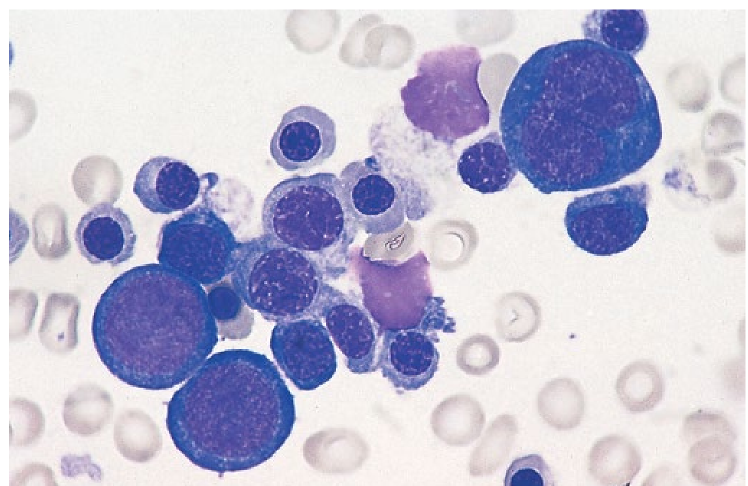
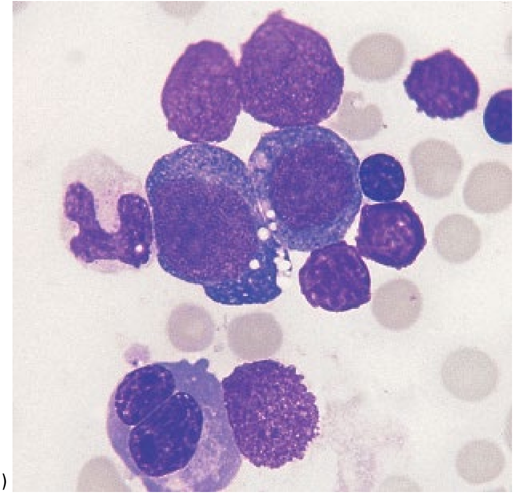


Fig. 1.41 BM film from a patient with FAB M6 AML showing marked erythroid hyperplasia but only mild dyserythropoiesis; one binucleated erythroblast is present. MGG × 100.



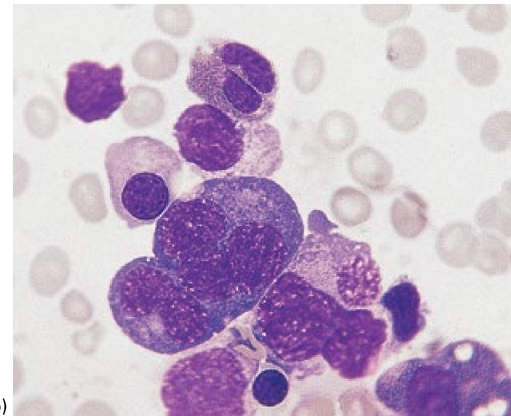


Fig. 1.42 BM film from a patient with FAB M6 AML showing: (a) a binucleated erythroblast and two vacuolated erythroblasts; (b) a giant multinucleated erythroblast. MGG × 100.

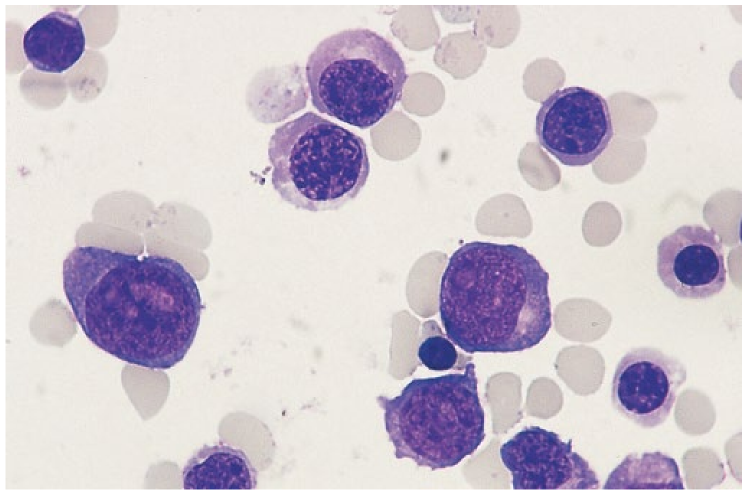
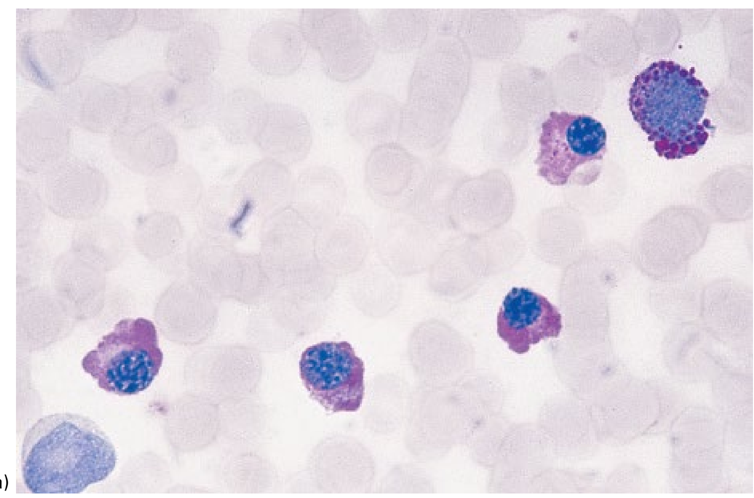


Fig. 1.43 A cytospin preparation of BM cells of a patient with FAB M6 AML showing late erythroblasts and three undifferentiated blasts. A positive reaction of the blast cells with a McAb to glycophorin A in this patient showed that these were primitive erythroid cells. MGG × 100.

erythroblasts. Dysplasia is not confined to the erythroid lineage, trilineage myelodysplasia being present in around half of patients [75–77]. 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 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 for such


(a)

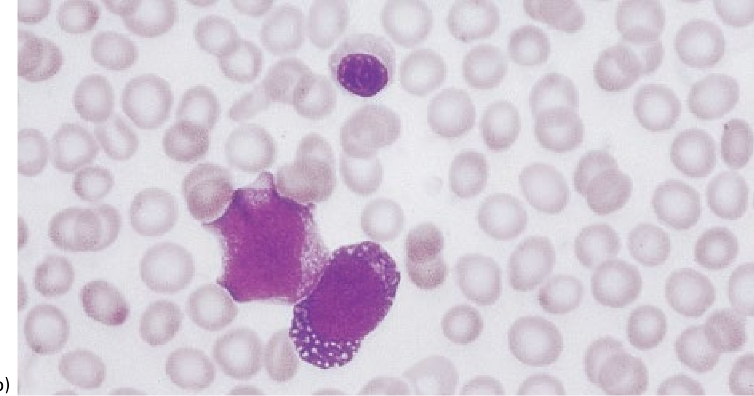

(b)

Fig. 1.44 (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.

cases (which are rare except when AML occurs in Down syndrome [78]) to be assigned to the FAB M6 category. Domingo-Claros *et al.* [75] found only 2 of 62 cases of erythroleukaemia to have these characteristics. Use of the terms M6 variant or 'pure erythraemia' is appropriate [75,77,79].

Overall the M6 category accounts for about 3–4% of cases of AML. The frequency is higher in the elderly [80]. Prognosis appears to be worse than for AML in general [37,81]. 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 [76], but in another both had an equally bad prognosis [77].

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 [76,82].

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. 1.44). Hayhoe and Quaglino [12] described a characteristic

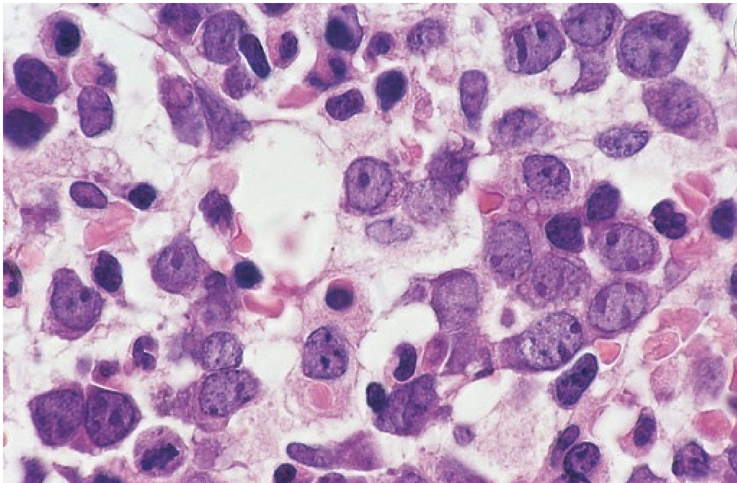


Fig. 1.45 BM trephine biopsy section in FAB M6 AML showing primitive erythroid cells, which can be distinguished from granulocyte precursors by their linear nucleoli, some of which abut on the nuclear membrane, and by their more basophilic cytoplasm (readily apparent on this Giemsa stain but not so apparent on an H&E stain). Paraffin embedded, Giemsa × 100.

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 [26]; they are usually positive for ANAE and ANBE [12]. 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 [12]. A Perls stain for iron may show coarse siderotic granules; in a minority of cases numerous ring sideroblasts are present.

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 [7] (Table 1.10). This category represents only about 1% of cases of AML in adults but 2–15% of childhood cases [83]. M7 AML shows a markedly increased incidence in children with Down syndrome (see page 202). In infants and children M7 AML may be associated with t(1;22)(p13.3;q13.1) (see page 180) and with other recurring cytogenetic abnormalities [83] including t(9;11)(p22;q23) [84], and in adults, and to a lesser extent in children, a significant proportion of cases are associated with abnormalities of chromosome 3q21.3q26.2, with loss or long arm deletion of chromosome 5 or 7 [85]. Children may have gain of chromosome 21, 19 or 8 [84]. 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 [85]. *GATA1* mutations are invariably present in Down syndrome-associated cases but are

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

also sometimes seen in other cases [86]. 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 [86]. 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 [87]. There is often an antecedent haematological abnormality, and in one series of patients 19% were therapy related [87].

Leukaemic megakaryoblasts are often highly pleomorphic. Prominent and multiple nucleoli and cytoplasmic basophilia have been noted [26]. Binuclearity and clumping of blast cells have been noted to be frequent features [88]. Rarely there is erythrophagocytosis or haemophagocytosis [71,89] or 'cannibalism' of leukaemic blast cells [90]. 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 [86]. 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 [91]. 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. 1.46) or ultrastructural cytochemistry (Fig. 1.47). The clinicopathological picture designated acute myelofibrosis, that is, pancytopenia with bone marrow fibrosis, usually represents acute megakaryoblastic leukaemia. There may be osteosclerosis as well as bone marrow fibrosis [92]. 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 1.48 and 1.49. Some cases show some maturation to dysplastic megakaryocytes, as is shown in Fig. 1.50. With the exception of cases among children with Down syndrome, the prognosis in both children and adults appears to be poor [37,88,91].

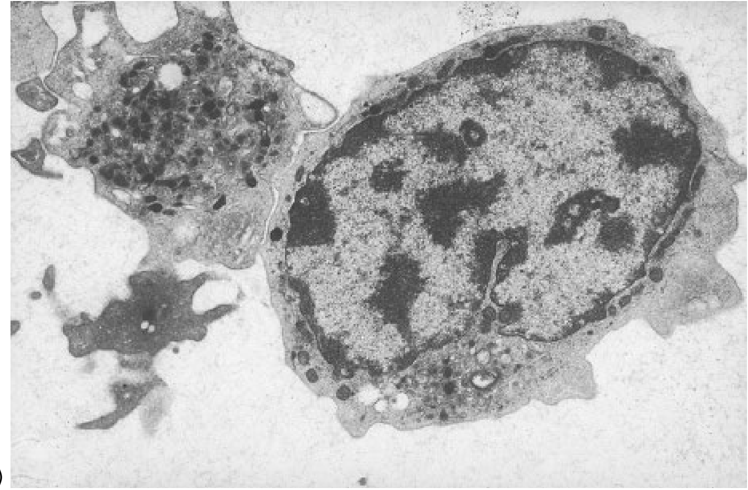
Cytochemical reactions in M7 acute myeloid leukaemia

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 [88]. 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. 1.50). Esterase activity is usually multifocal punctate [88] but is sometimes localized to the Golgi zone [30,88]. There is a similar localization of acid phosphatase activity, which is tartrate sensitive [26]. 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, for example M2Eo and M4Eo. Such cases may have cardiac and other tissue damage as a result 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 eosinophilic differentiation [93].

(a)



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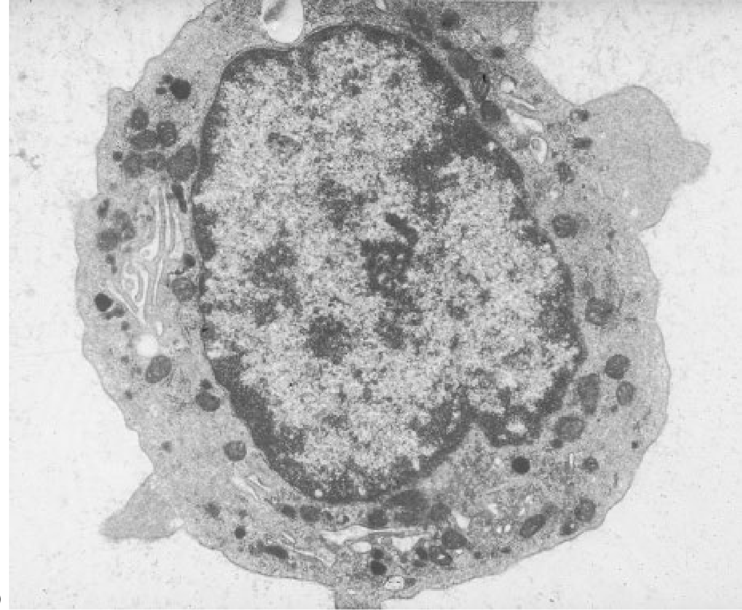


Fig. 1.46 Ultrastructural examination of peripheral blood cells from a patient with megakaryoblastic transformation of chronic myeloid leukaemia showing: (a) a blast cell and a giant platelet; the megakaryoblast has characteristic granules including several bull's eye granules; (b) a megakaryoblast with platelet demarcation membranes. (With thanks to Professor Daniel Catovsky.)

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.1.51). Mature eosinophils often show vacuolation, degranulation and nuclear hyper- or hypolobation. 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. 1.52). Occasionally similar crystals are seen within leukaemic cells (Fig. 1.53).

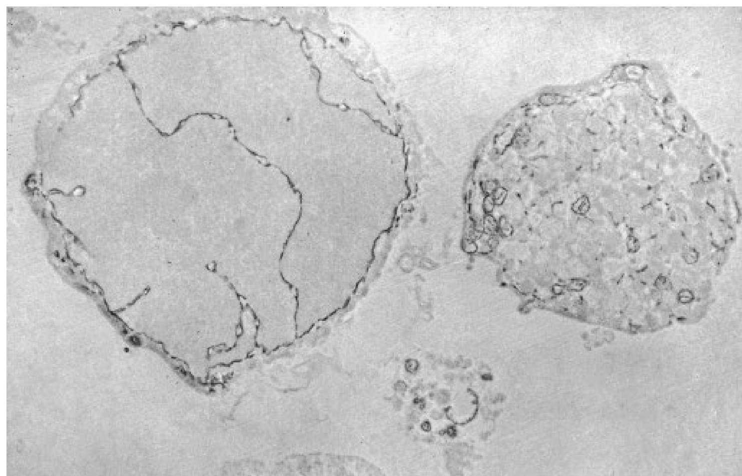


Fig. 1.47 Ultrastructural cytochemistry of a blast cell showing a positive platelet peroxidase reaction. (With thanks to Professor Daniel Catovsky.)

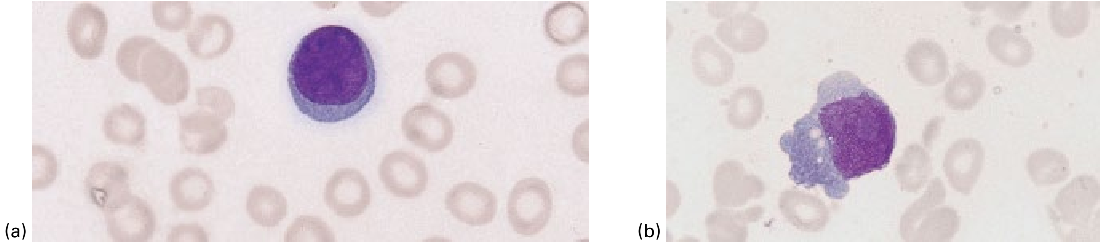


Fig. 1.48 PB and BM films from a patient with FAB M7 AML presenting as acute myelofibrosis; the nature of the leukaemia was demonstrated by a positive reaction for platelet peroxidase. (a) PB film showing mild anisocytosis and a blast cell with no distinguishing features. (b) BM film showing a megakaryoblast. MGG × 100.

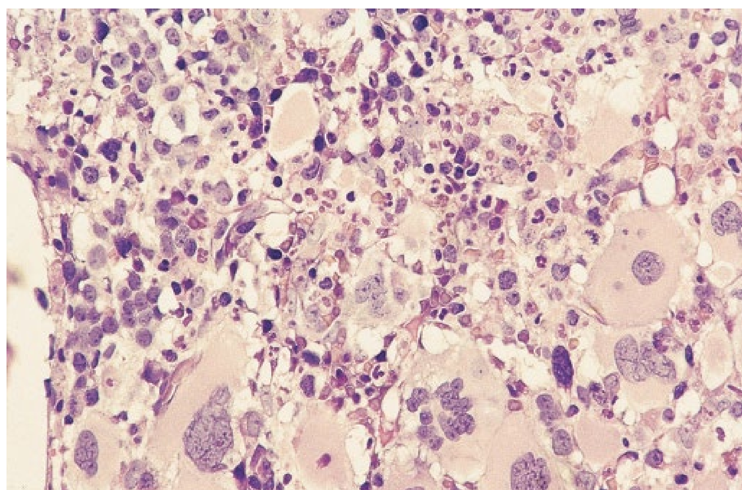


Fig. 1.49 Trephine biopsy section from a patient with FAB M7 AML showing increased blasts and large dysplastic megakaryocytes. Paraffin embedded, H&E × 40.

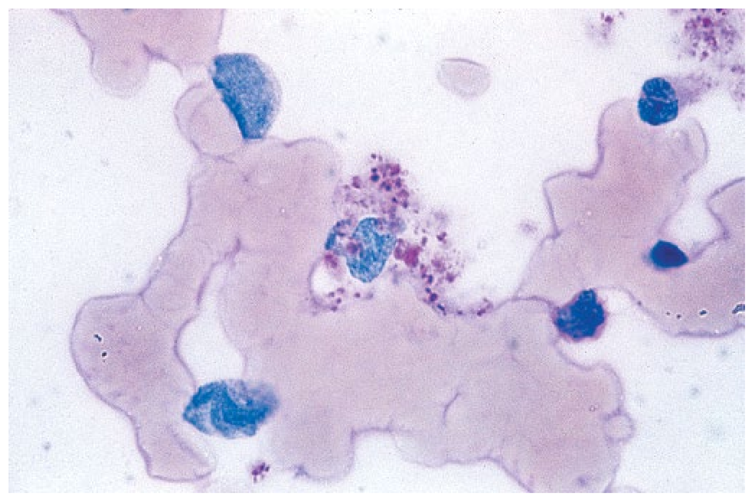


Fig. 1.50 BM film in FAB M7 AML showing a micromegakaryocyte with cytoplasmic blebs, which are PAS positive. PAS × 100.

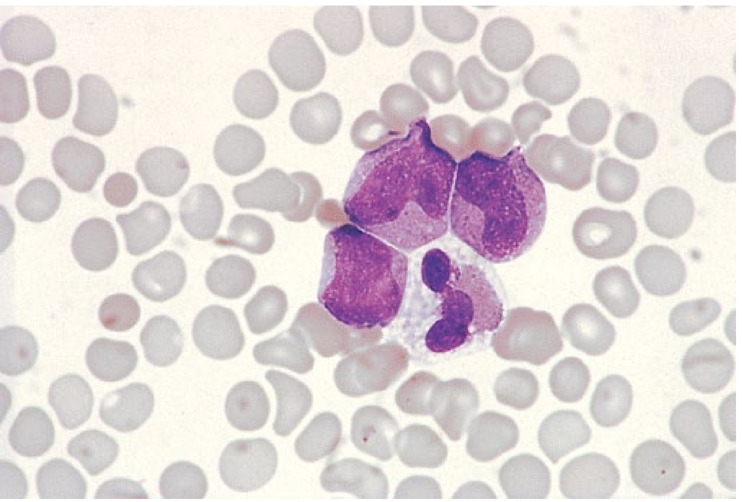


Fig. 1.51 PB film in acute eosinophilic leukaemia with abnormal eosinophil precursors showing a mixture of eosinophilic and azurophilic granules; maturing eosinophils are degranulated and some have nuclei of bizarre shapes. MGG × 100. (With thanks to Dr Alistair Smith, Southampton.)

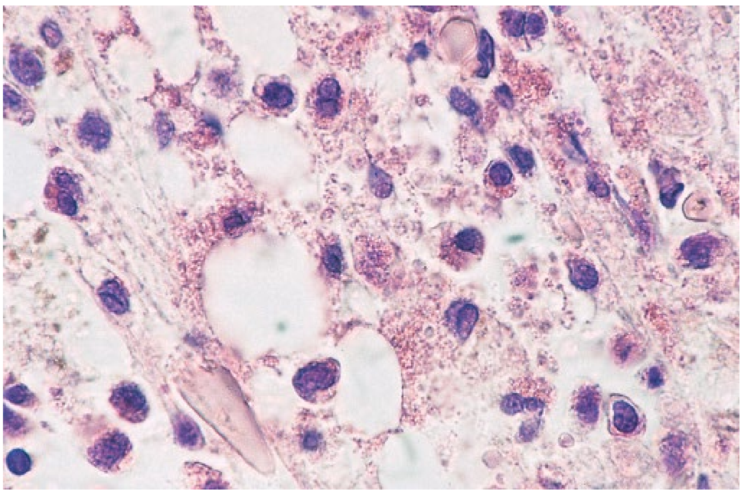


Fig. 1.52 Trephine biopsy section in acute eosinophilic leukaemia showing numerous eosinophils and part of a Charcot–Leyden crystal (same patient as Fig. 1.51). Paraffin embedded, H&E × 100. (With thanks to Dr Alistair Smith and Dr Bridget Wilkins, London.)

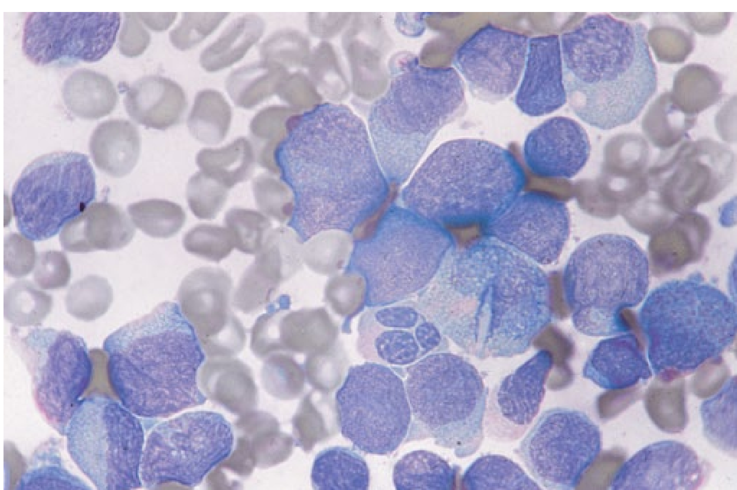


Fig. 1.53 BM film from a patient with acute eosinophilic leukaemia showing a Charcot–Leyden crystal within a leukaemic cell. MGG × 100.

Cytochemical reactions 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 [69]. Cells of the eosinophil lineage are usually CAE negative but positively staining granules may be seen in acute eosinophilic leukaemia [67]. A combined cytochemical stain for CAE and cyanide-resistant peroxidase activity is a convenient means of distinguishing cells of neutrophil and eosinophil lineage [69].

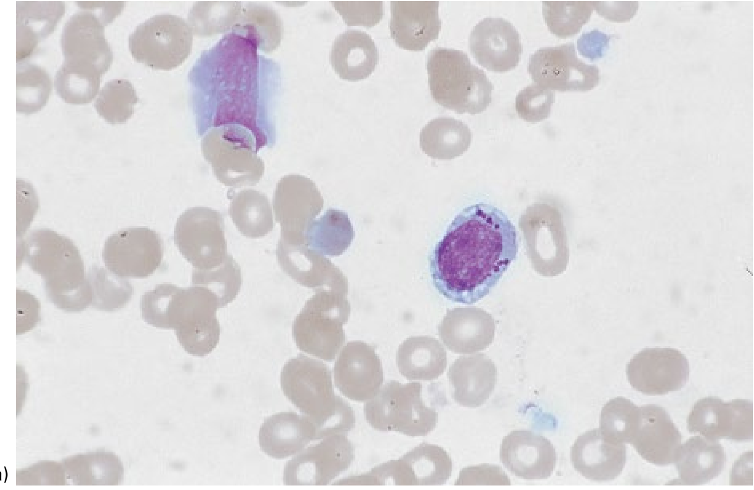
Acute basophilic leukaemia

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 [94]. Patients with acute basophilic leukaemia do not usually show features of histamine excess [95] but some patients have had urticaria, peptic ulceration or other gastrointestinal disturbance [96], and anaphylactoid reactions can occur following chemotherapy [97].

In cases with maturation, basophils are usually easily recognized by their cytological and cytochemical characteristics (Fig. 1.54). 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 [97]. Blasts of basophil lineage may contain Auer rods [98].

Cytochemical reactions in acute basophilic leukaemia

In acute basophilic leukaemia without maturation [99], 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


(a)

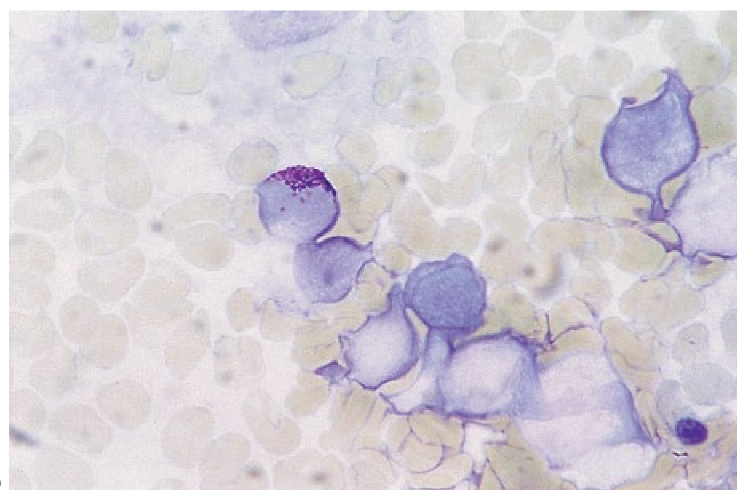

(b)

Fig. 1.54 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.

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 [94]. ε-amino caproate activity [100] is specific for the basophil lineage. At an ultrastructural level, ruthenium red can be used to identify basophil granules [99].

Acute mast cell leukaemia

Mast cell leukaemia can occur either *de novo* (three-quarters of cases) or as the terminal phase of systemic mastocytosis (one-quarter of cases) [101]. Uncommonly, it follows paediatric mastocytosis, sometimes after an interval of many decades [101]. Clinical features often include hepatomegaly and splenomegaly but lymphadenopathy, present in 37% of cases, is more common than in other types of acute myeloid leukaemia [101]. Skin infiltration is seen mainly, but not only, in those with previous systemic mastocytosis [101]. Mast cell activation symptoms (e.g. fever, flushing, diarrhoea and palpitations) are common. Mast cell leukaemia 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 not only are the majority of cases

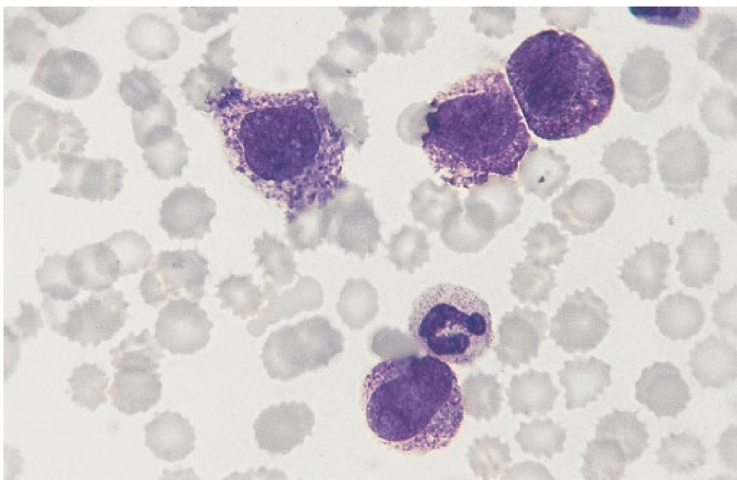


Fig. 1.55 PB film in mast cell leukaemia showing a neutrophil and four mast cells. MGG × 100. (With thanks to Dr Ian Bunce and Miss Desley Scott, Brisbane.)

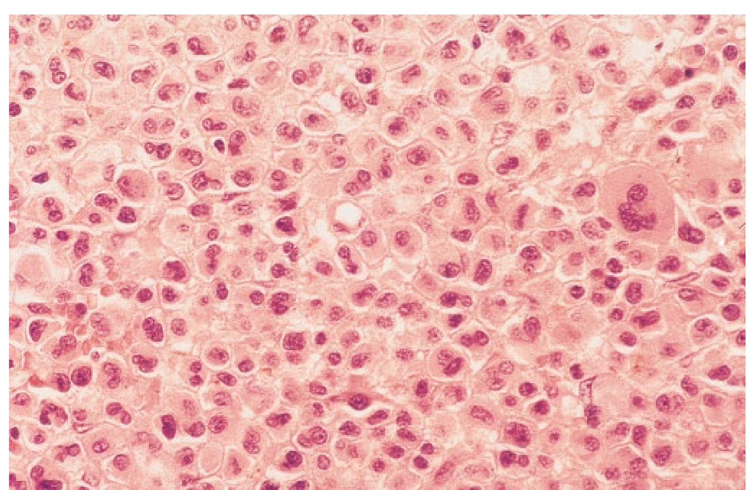
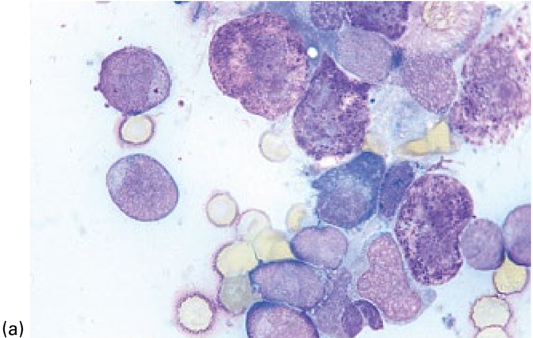


Fig. 1.56 Trephine biopsy section from a patient with acute mast cell leukaemia showing neoplastic mast cells with irregular nuclei and voluminous cytoplasm. Paraffin embedded, H&E × 40. (With thanks to Professor Ghulam Mufti, London.)

de novo, but also systemic mastocytosis terminates in other types of AML more often than in mast cell leukaemia; in one such patient, with acute erythroid leukaemia, a *KIT* D816V mutation was demonstrated both in mast cells and in leukaemic blasts and erythroid cells [102]. The peripheral blood may show mast cells (Fig. 1.55), which are often immature or morphologically abnormal with hypogranularity or nuclear lobulation; however, about 60% of cases are aleukaemic [101]. The bone marrow is hypercellular and infiltrated by mast cells (Fig. 1.56). The neoplastic mast cells can include granular and agranular blast cells, promastocytes and mast cells; all express mast cell tryptase. Ultrastructural examination can confirm the diagnosis but it should be noted that in some cases cells show both basophil and mast cell characteristics [103]. As for acute basophilic leukaemia, anaphylactoid reactions may follow chemotherapy [104]. In some patients there is differentiation to both myeloblasts and mast blasts (Fig. 1.57) [105]. The term 'myelomastocytic leukaemia' has been suggested for these cases [106]. Mutations in the *KIT* gene are found, including, but not only, the D816V mutation characteristic of systemic mastocytosis, which was reported in


(a)

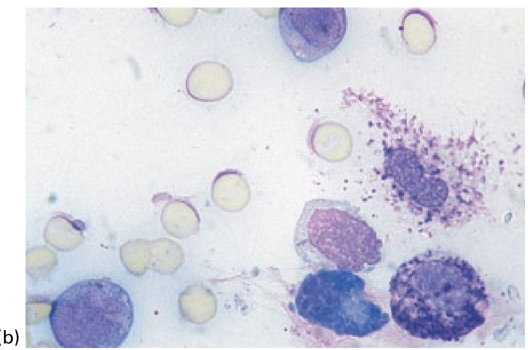

(b)

Fig. 1.57 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. (With thanks to Dr Neelam Varma, Chandigar.)

13/28 cases (46%) in one series [101]; intragenic deletions have also been demonstrated [107]. Reported cytogenetic abnormalities include del(5q), t(10;16)(q22;q13q22) and t(8;21)(q22;q22.1) [101].

Immunophenotype in acute mast cell leukaemia

There may be expression of CD25 (75%) or CD2 (52%) plus markers of immaturity such as CD34, HLA-DR and CD123 [101]. CD30 may be expressed [108].

Cytochemical reactions 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. 1.58) [105]. They also express CD117.

Langerhans cell leukaemia

Rare cases of AML have leukaemic cells showing features of Langerhans cells [109] (Fig. 1.59). Such cases may occur *de novo* but it is likely that cases resembling M5 AML supervening in Langerhans cell histiocytosis [110] also represent a leukaemia

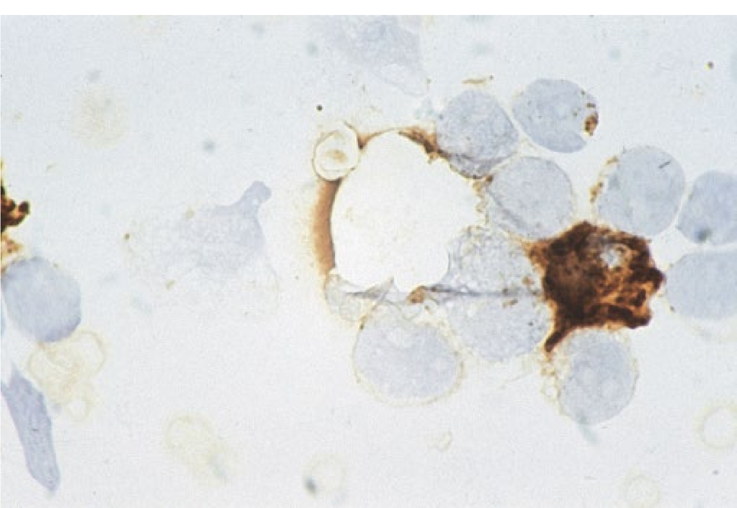


Fig. 1.58 BM film from a patient with acute mast cell leukaemia showing a mature mast cell packed with granules that are strongly positive for mast cell tryptase and several blast cells with tryptase-positive granules; these latter cells are therefore identified as blast cells of mast cell lineage (same patient as Fig. 1.57). Immunoperoxidase × 100. (With thanks to Dr Neelam Varma and Dr Bridget Wilkins.)

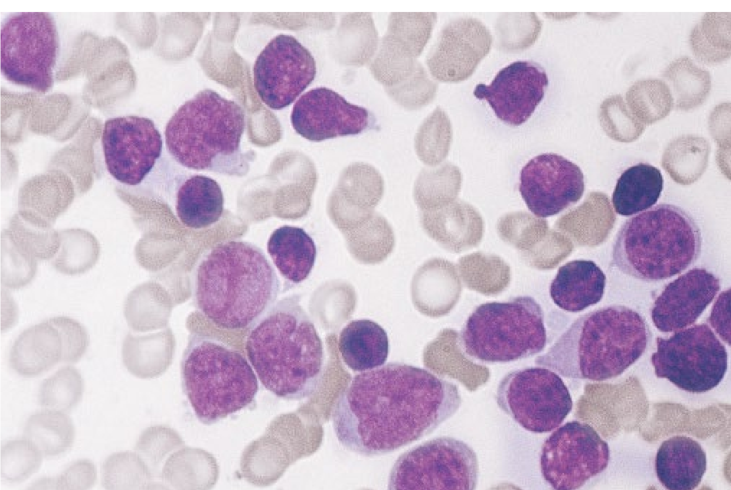


Fig. 1.59 Langerhans cell leukaemia. MGG×100. (With thanks to Dr B.I.S. Srivastava, Buffalo, New York.)

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% [111], less than 40% [112], less than 30% [113] or, in the WHO classification, less than 20% [114]. Hypoplastic AML can occur *de novo* or supervene in MDS. Often 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, using FAB criteria, 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 [115]. 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 [115].

Clinical correlates of FAB categories of acute myeloid leukaemia

The FAB category of M3 AML is a distinct disease entity. 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 [116] 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, ALL 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

Table 1.11 Morphological features of acute lymphoblastic leukaemia 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

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 or T lineage.

The FAB group have assigned ALL to three cytological categories: L1 (70–80% of childhood cases); L2 (about a quarter of childhood cases); and L3 (1–2%). The classification is summarized in Table 1.11 and illustrated in Figs 1.60–1.69). The only clinical significance of the FAB classification of ALL is that (i) cases with L1 cytological features are highly likely to be ALL, which can be useful in a resource-poor setting (whereas cases with L2 cytological features may represent M0 AML); and

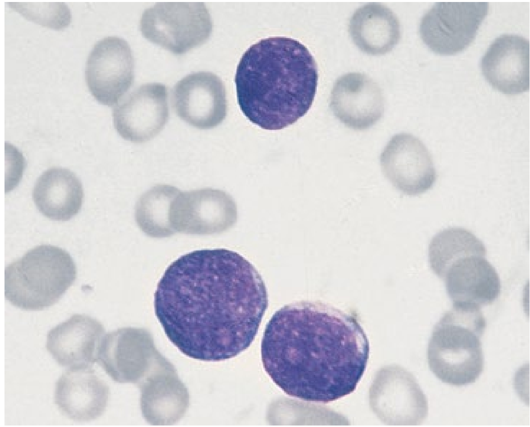


Fig. 1.60 PB film of a patient with FAB L1 acute lymphoblastic leukaemia (ALL). MGG × 100.

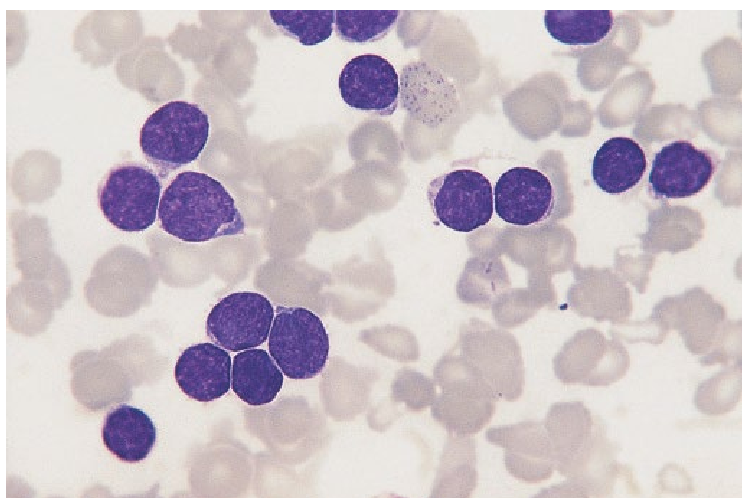


Fig. 1.61 BM film from a patient with FAB L1 ALL. MGG × 100.

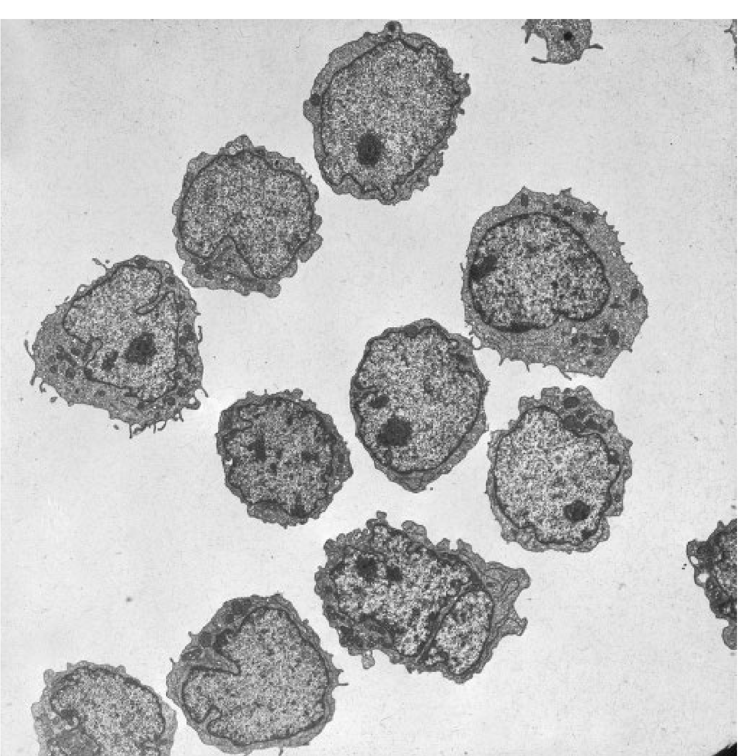


Fig. 1.62 Ultrastructure of lymphoblasts in FAB L1 ALL. (With thanks to Professor Daniel Catovsky.)

(ii) that the rapid recognition of L3 cytological features is important because this is usually associated with a mature B immunophenotype and represents a leukaemic phase of Burkitt lymphoma or, occasionally, transformation of a low-grade lymphoma, such as follicular lymphoma, as the result of the occurrence of a Burkitt lymphoma-related translocation occurring as a second event [117]. Neither of these groups of cases is any longer classified as ALL.

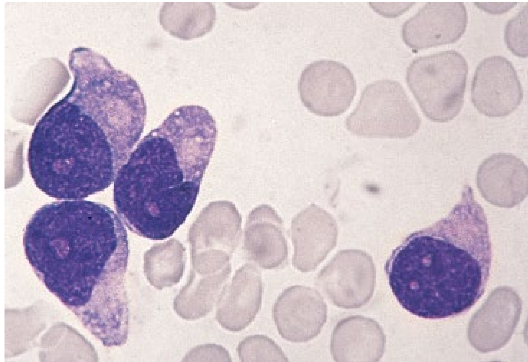


Fig. 1.63 BM film from a patient with FAB L2 ALL showing large pleomorphic blasts; the cells were CD10 positive. MGG × 100.

Cytological features of L1 and L2 ALL are described in detail in the previous edition of this book. L1 type lymphoblasts are medium sized with a fairly regular nucleus, high nucleocytoplasmic ratio, a diffuse chromatin pattern and small and inconspicuous nucleoli. L2 type lymphoblasts may be larger and are more pleomorphic. Because L3 cytological features retain clinical significance, they are described in more detail below.

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

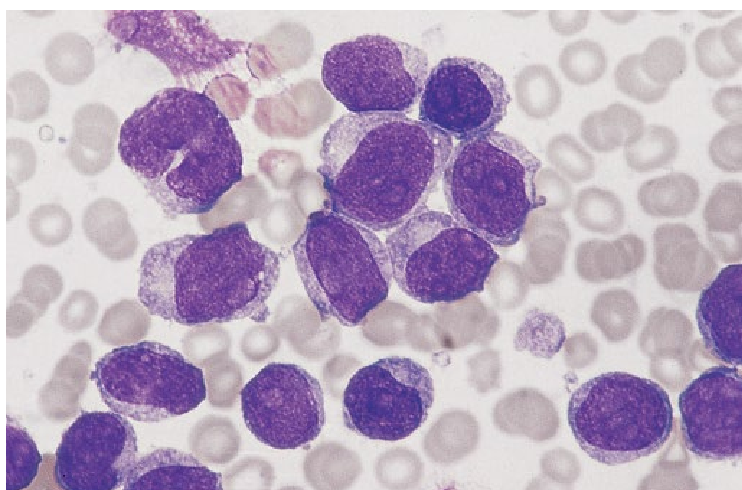


Fig. 1.64 BM film from a patient with FAB L2 ALL showing medium to large pleomorphic blasts, which were CD10 negative but positive for CD19, HLA-DR and TdT. MGG × 100.

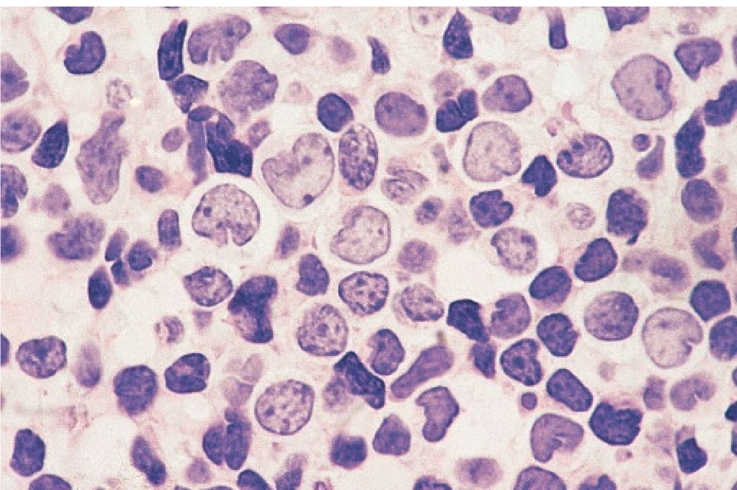


Fig. 1.65 Trephine biopsy section from a patient with FAB L2 ALL. Resin embedded, H&E × 100.

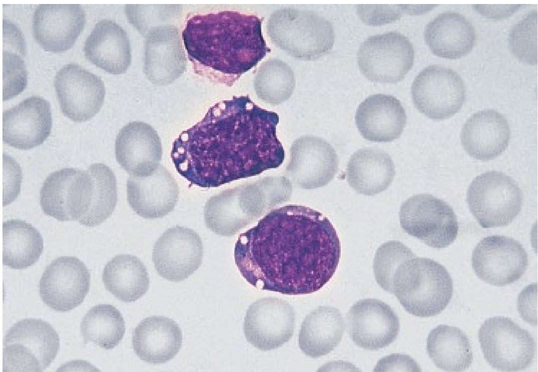


Fig. 1.66 PB film of a patient with FAB L3 ALL with the immunological phenotype being mature B cell. MGG × 100.

relatively mature immunophenotype, have leukaemic cells that are difficult to recognize as blasts because of chromatin condensation and inconspicuous nucleoli [118]; immunophenotyping is of importance in these cases. Occasionally leukaemic lymphoblasts have cup-shaped nuclei [119] (Fig. 1.70), a feature that is much more common in AML. In a minority of cases there are small numbers of azurophilic granules. Sometimes granules are prominent. Occasional patients have lymphoblasts with coarse purple granules [67]. Rarely there are structures resembling Auer rods [120].

When immunophenotyping is available, cytochemical reactions are redundant in ALL. Their role is described in Chapter 8.

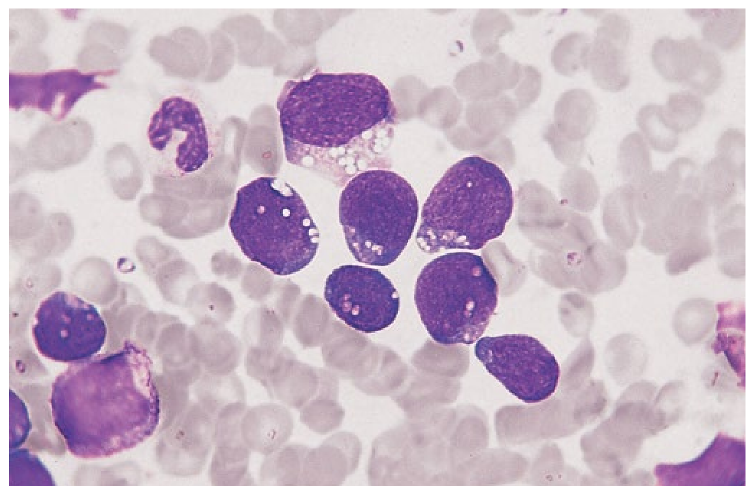


Fig. 1.67 BM film of a patient with FAB L3 ALL with the immunological phenotype being mature B cell. MGG × 100.

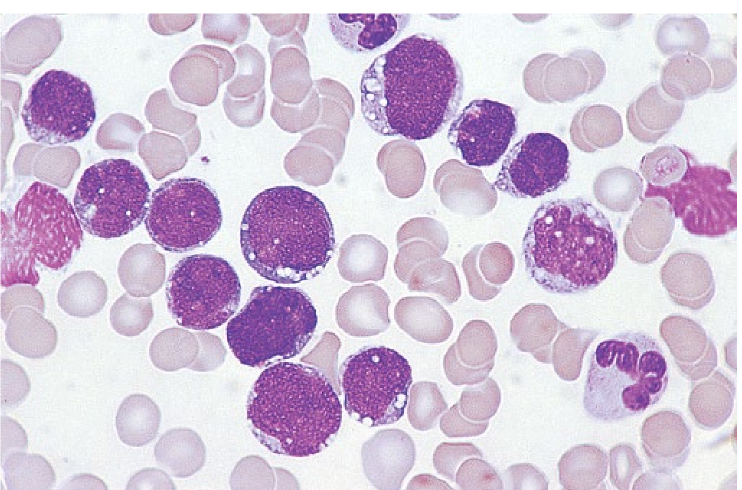


Fig. 1.68 PB film a case of FAB L3 ALL, which was unusual in being of T lineage and having a t(7;9) translocation. MGG × 100.

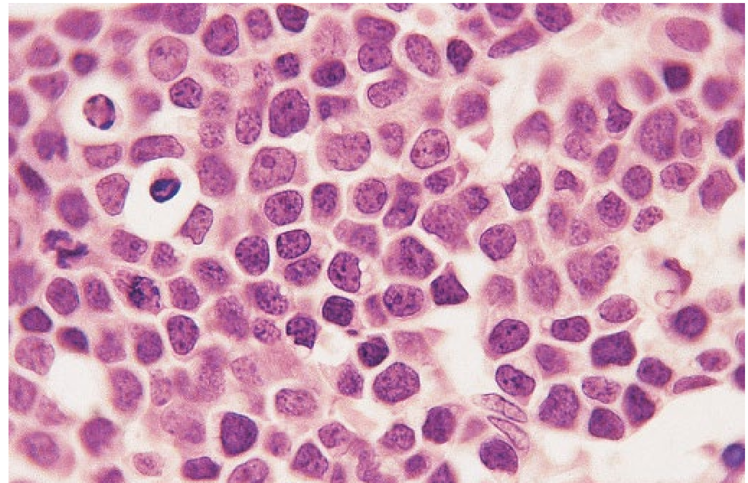


Fig. 1.69 Trephine biopsy section from a patient with FAB L3 ALL, B-cell phenotype; vacuolation of some of the blasts can be observed and there are two blasts undergoing apoptosis. Paraffin embedded, H&E × 100.

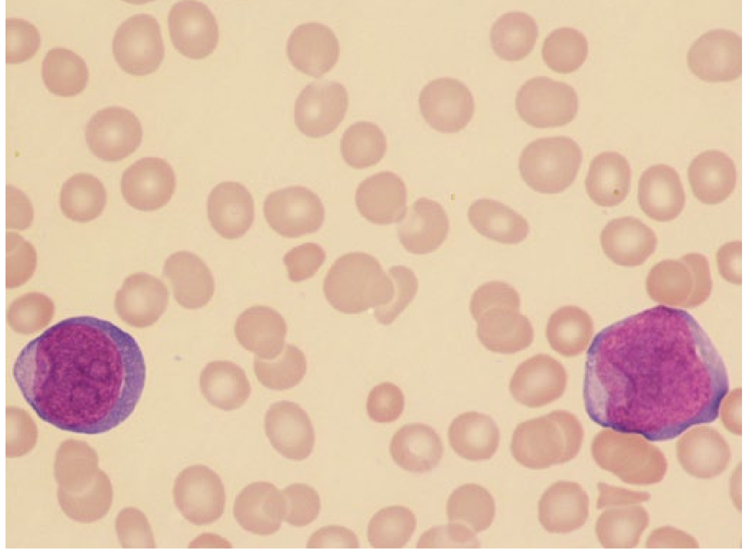


Fig. 1.70 PB film of a patient with B-lineage ALL showing two cup-shaped blast cells; this feature is much more common in AML with *NPM1* and *FLT3*-ITD mutations than in ALL.

'Acute lymphoblastic leukaemia' of L3 subtype

In L3 'ALL' [9] the blast cells are medium to large and homogeneous. There is a moderate amount of cytoplasm. 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. The mitotic index is high and many apoptotic cells are seen. The cytoplasm is strongly basophilic with variable but prominent vacuolation. Typical examples are shown in Figs 1.66–1.69.

A minority of cases with L3 cytological features have a common ALL phenotype, a pre-B immunophenotype (cytoplasmic immunoglobulin positive) [121] or even a T-cell [122] (Fig. 1.68) phenotype; such cases continue to be classified as ALL. Rarely the immunophenotype is of MPAL [123] including hybrid B–T [124] or B-myeloid [125]. Cases have also been reported with a lack of B or T markers but with the characteristics of very early erythroid cells [126, 1257; as these latter cases had cytogenetic findings usually associated with Burkitt lymphoma the involvement of a primitive cell with the potential for both B lymphoid and erythroid differentiation is suggested. Rarely L3 cytology is associated with small cell carcinoma of the lung [128] or undifferentiated carcinoma [129].

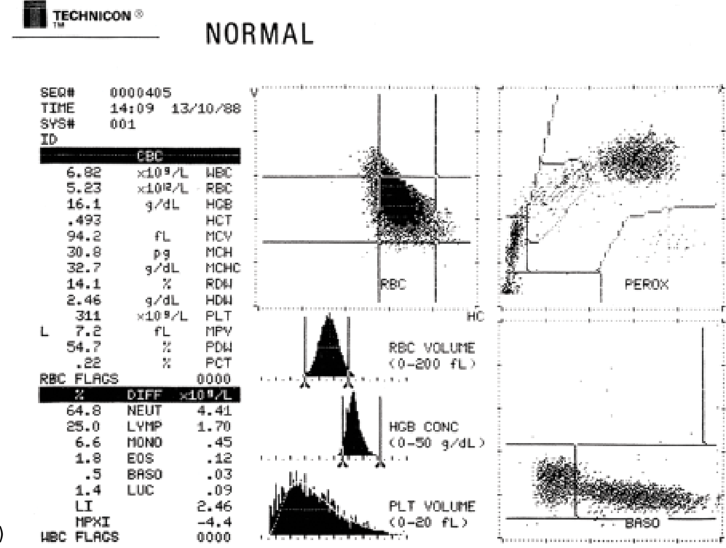
When a patient shows L3 cytological features, further investigation is essential and is urgent; immunophenotyping and cytogenetic or molecular genetic analysis are required. Patients with L3 cytology and a mature-B immunophenotype may have not only Burkitt lymphoma-related translocations but also t(14;18)(q32;q21.3); the prognosis of the latter group is poor and optimal management has not been defined. Rare cases with a B-cell precursor immunophenotype have t(9;22)(q34.1;q11.2) or t(1;19)(q23;p13.3) and should be classified and treated as ALL.

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.

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 [130] (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 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 (Horiba Medical) employ Sudan black B instead of peroxidase cytochemistry and give similar information to Siemens/Bayer instruments. Other automated instruments, for example 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 [131].



(a)

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.

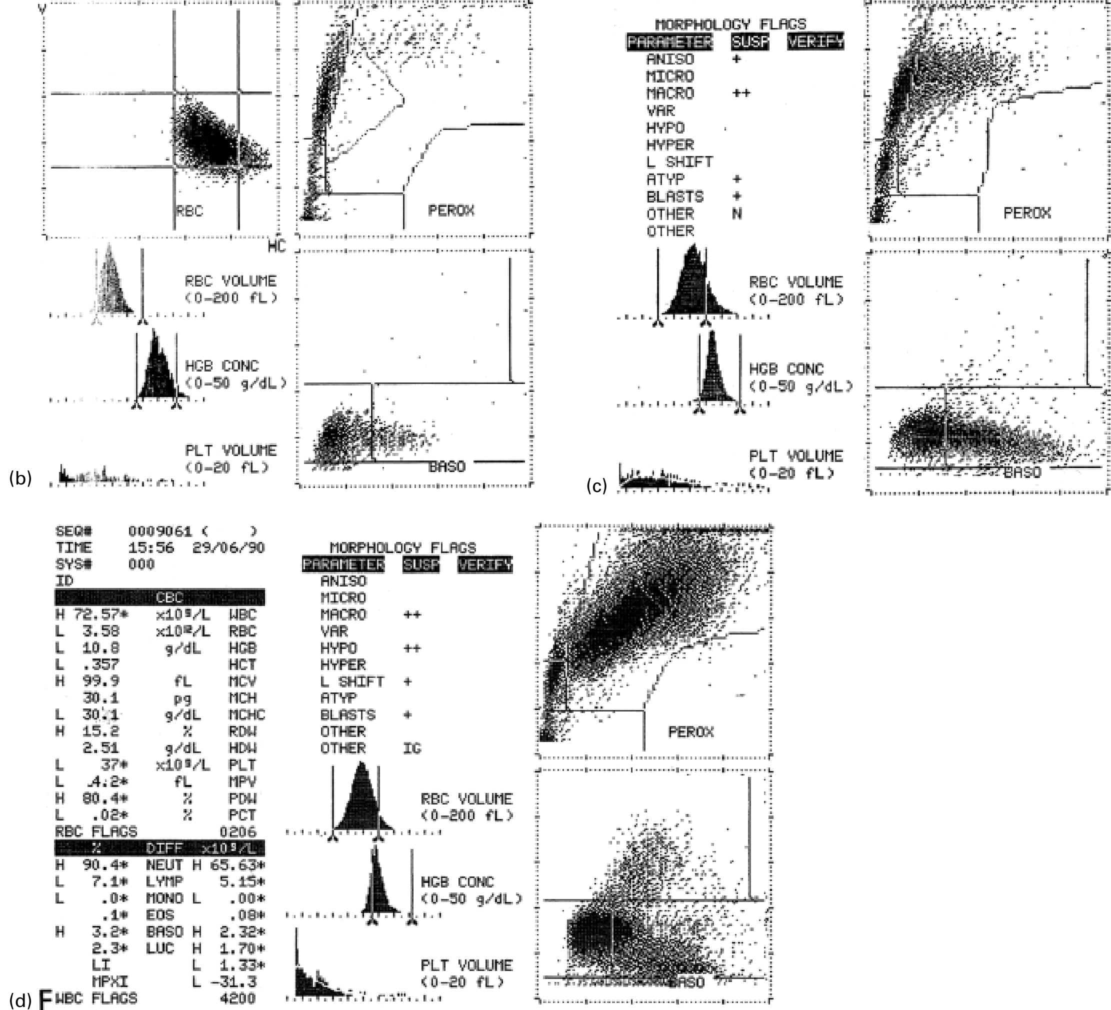


Fig. 1.71 (Continued) (b) Histograms, red cell cytogram and scatterplots on a blood sample from a patient with FAB M0 AML performed on a Bayer-Technicon H2 automated analyser. The blasts are peroxidase negative and therefore fall into the LUC area; the only indication that this is AML not ALL is that 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. (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.

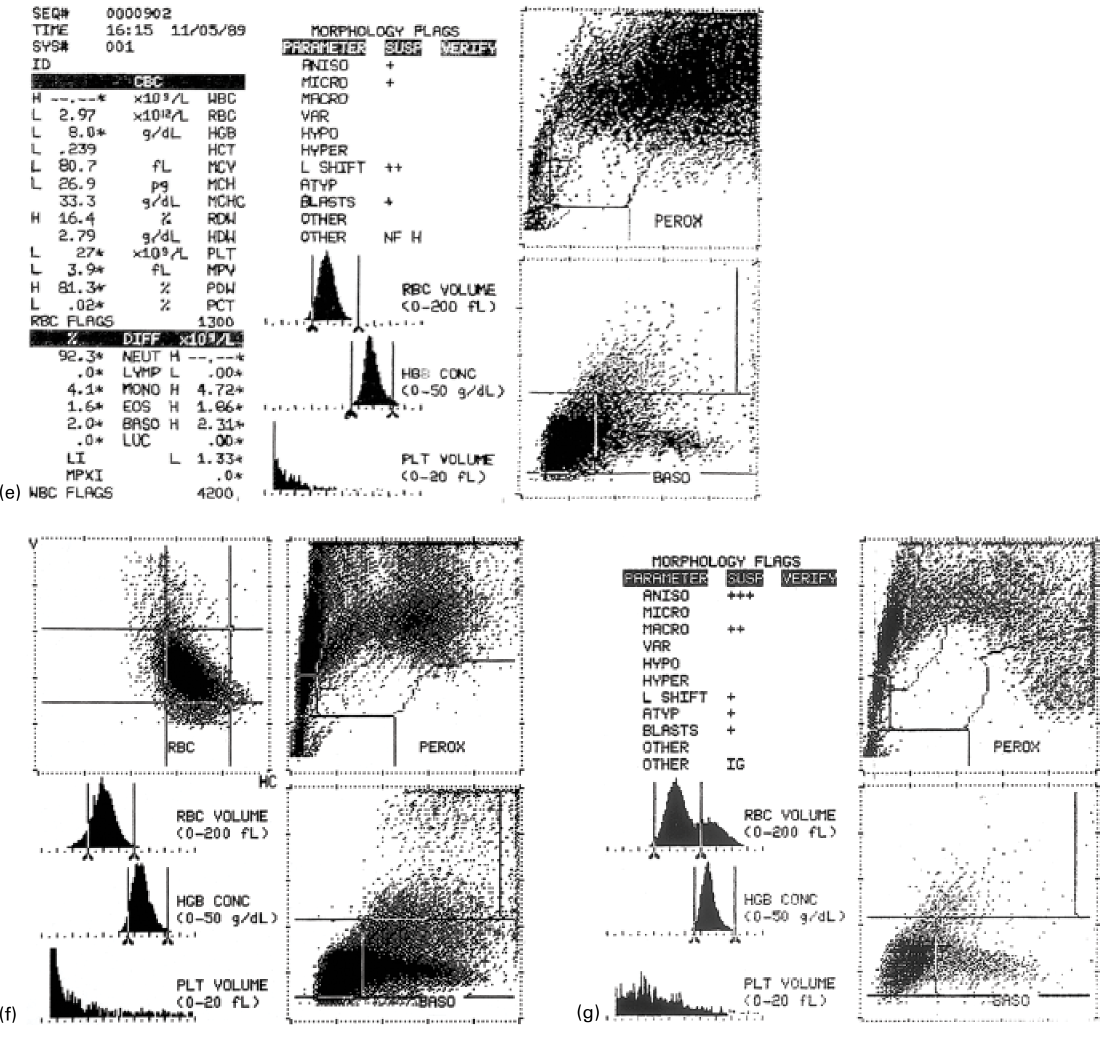


Fig. 1.71 (Continued) (e) Histograms and scatterplots on a blood sample from a patient with FAB M3V AML performed on a Bayer-Technicon H2 automated analyser. The abnormal promyelocytes are intensely peroxidase positive and form a triangular cluster based on the right-hand margin; there is pseudobasophilia and thrombocytopenia. The scattergrams in FAB M3 AML show the same features as are shown in this case of FAB M3V AML. (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 (cf. 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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2

Immunophenotyping and Cytogenetic/Molecular Genetic Analysis

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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.

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In the non-Hodgkin lymphomas (NHL) and chronic lymphoid leukaemias immunophenotyping is likewise a crucial diagnostic tool.

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.

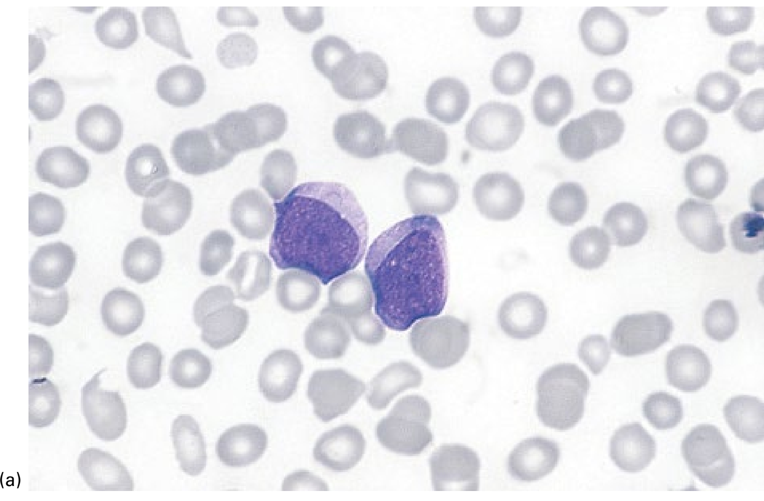
As immunophenotypic and genetic analysis have increased in importance, cytochemistry has become less important with its use now largely confined to acute myeloid leukaemia (AML), the myelodysplastic syndromes (MDS) and hairy cell leukaemia.

Immunophenotyping

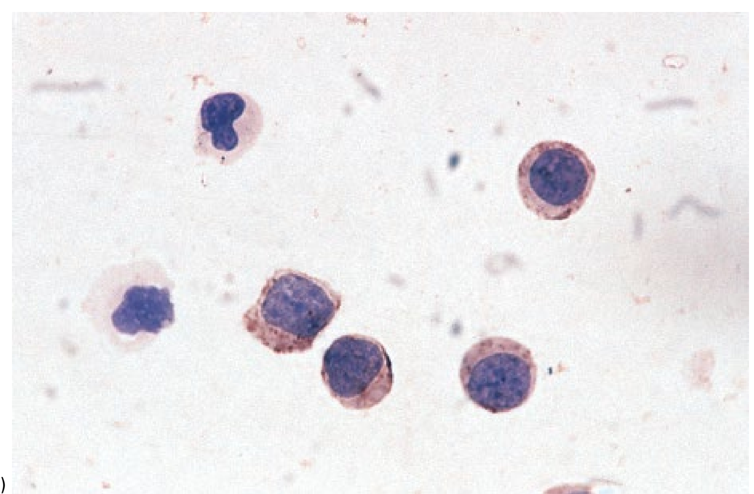
Leukaemic and normal cells of different types express characteristic nuclear, cytoplasmic and cell surface antigens. This is referred to as the immunophenotype of the cell. Characterization of the immunophenotype is referred to as immunophenotyping and is achieved by means of labelled antibodies that 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.



(a)



(b)

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.

Immunofluorescence, supplemented by light scatter measurements, is the basis of flow cytometric immunophenotyping [1–7]. Flow cytometry has the advantage over immunocytochemistry in 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 light scatter – both forward scatter (FSC) and side 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, which absorbs and is excited by light and then emits light of a longer wavelength as it returns to its unexcited state; light emitted is detectable at a wavelength specific for each fluorochrome. 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. FSC and SSC 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

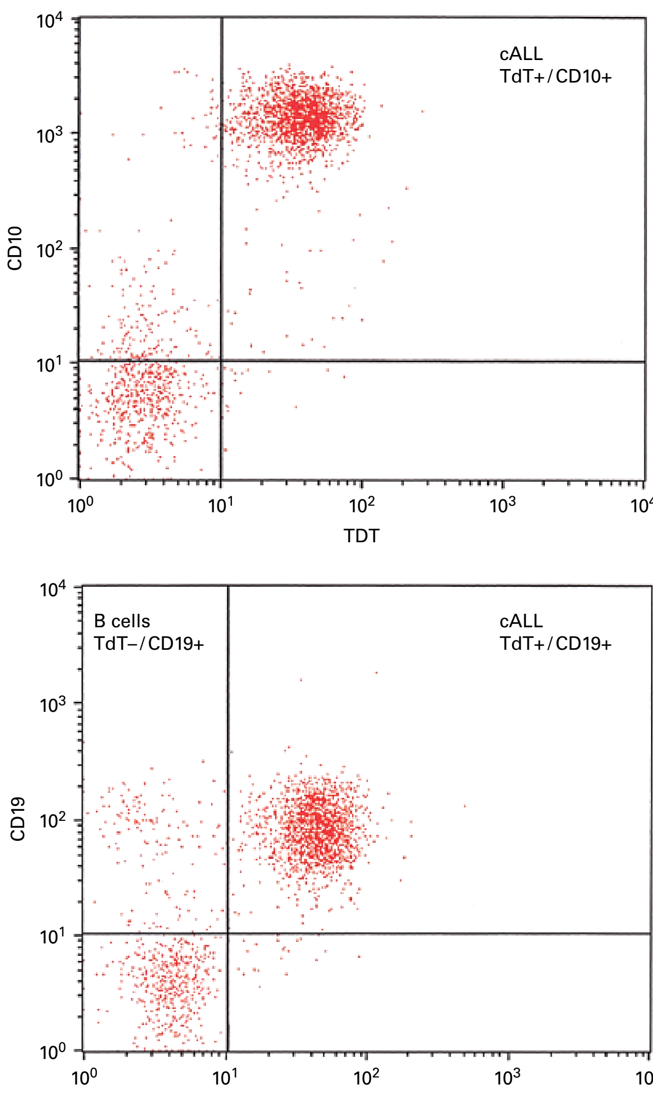


Fig. 2.2 Immunophenotyping of a case of acute lymphoblastic leukaemia (ALL) by flow cytometry with two-colour immunofluorescence: the upper scatterplot shows leukaemic cells that are positive for CD10 and terminal deoxynucleotidyl transferase (TdT), the cells that are negative for both being residual 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. (With thanks to Mr Ricardo Morilla, London.)

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, particularly the complexity of the nucleus. Cytoplasmic vacuolation affects both FSC and SSC. 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 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

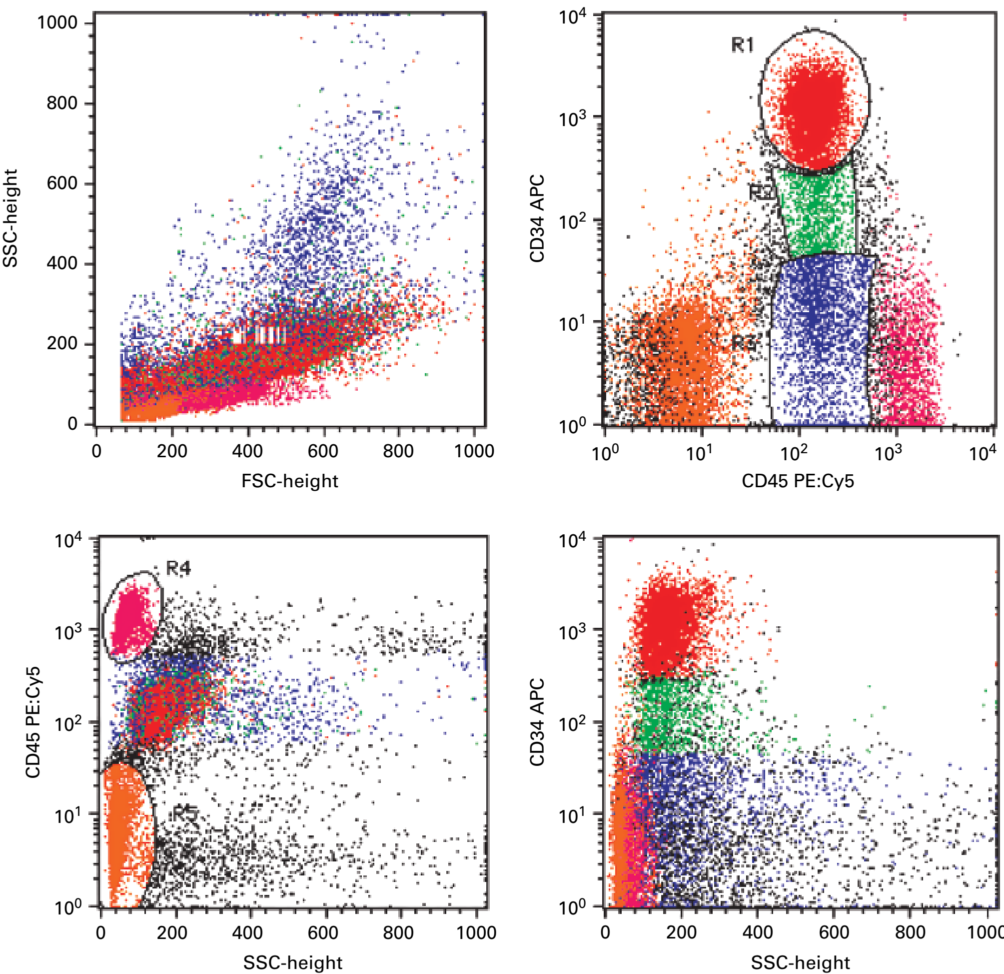


Fig. 2.3 Four-colour flow cytometric immunophenotyping showing characteristics of various types of cell. Standard analysis regions (R1–R3) are set to delineate stages of myeloid blast cell differentiation: R1, the most immature blast cells (CD34+++); R2, maturing blast cells (CD34+); R3, mature blast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

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 the cell is then permeabilized, permitting 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

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)	
Cy7 (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
Phycoerythrin + Cy5.5 tandem conjugate	PE-Cy5.5
Phycoerythrin + Cy7 tandem conjugate	PE-Cy7

dilution must be determined within the laboratory. For example, fluorochrome-labelled 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. Although monoclonal antibodies are generally preferred, 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 four to six-colour analysis has now become common and ten-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 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 expression of 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 acute promyelocytic leukaemia 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, for example in assessment of minimal residual disease (MRD), 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. TP53) and the expression of genes conveying multiple drug resistance to leukaemic cells (e.g. *MDR1*, *MRP1* and *LRP*). Techniques have also been developed using differentially labelled beads

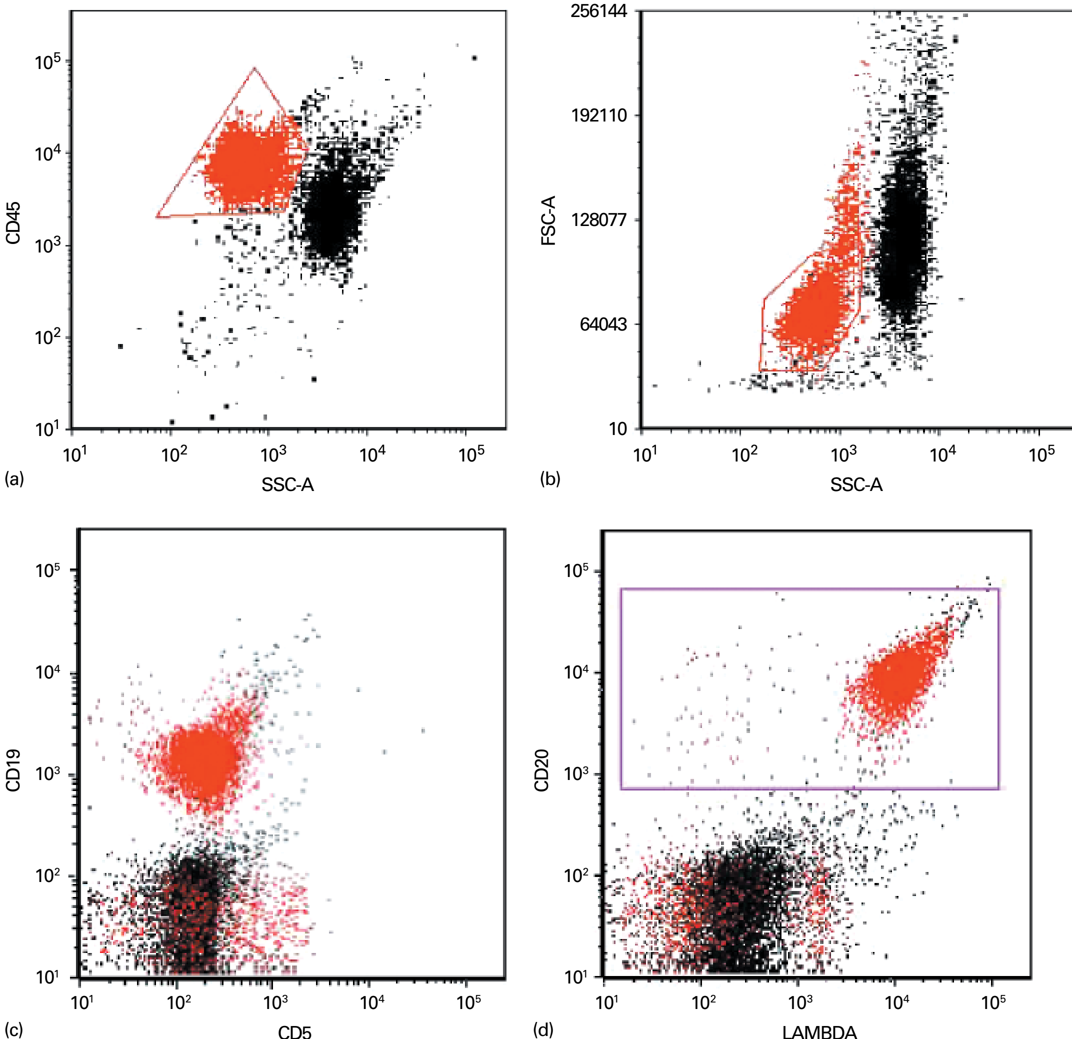


Fig. 2.4 Flow cytometric immunophenotyping in a patient with mantle cell lymphoma showing an abnormal population of B cells expressing CD19, CD5, λ and CD79b. (a, b) Plots of CD45 versus SSC and of forward scatter (FSC) versus SSC identified two populations of cells; the cluster painted red in these 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.) (With thanks to Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

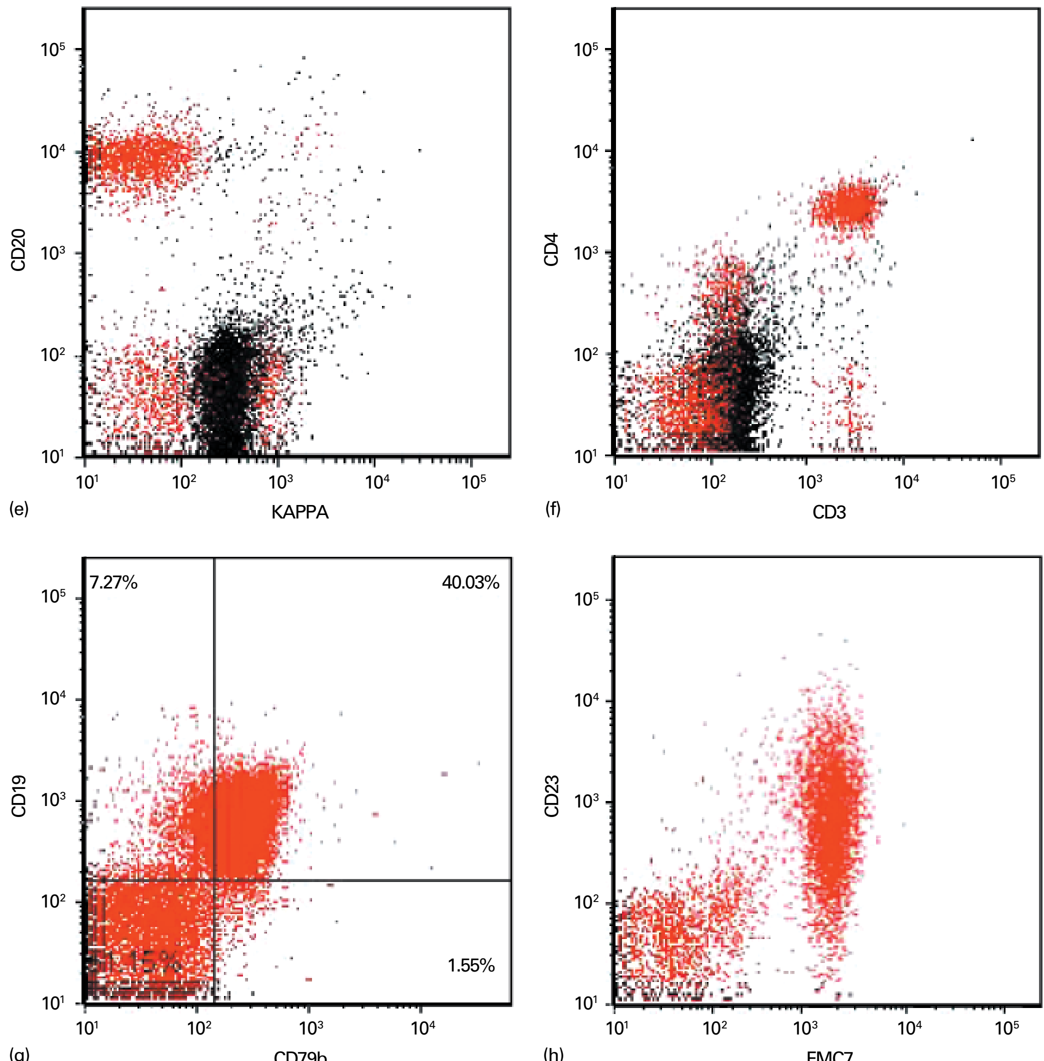


Fig. 2.4 (Continued)

to capture fusion proteins and ectopically expressed or overexpressed proteins in leukaemic cell lysates, by means of monoclonal antibodies bound to beads. This technique can indicate the likelihood of specific genetic abnormalities more rapidly than is possible with molecular techniques.

Flow cytometric 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 techniques in order to cope with the workload 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

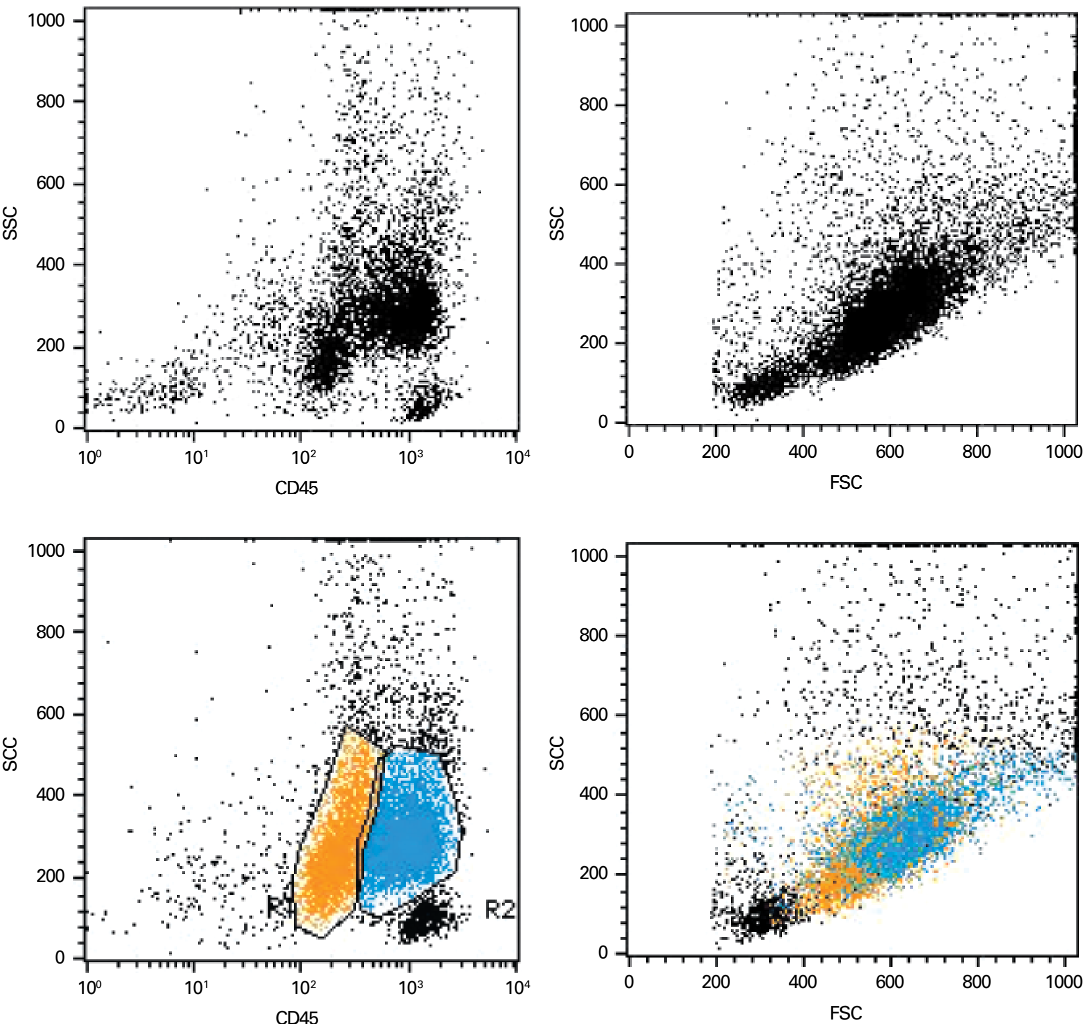


Fig. 2.5 Dot plots of flow cytometric immunophenotyping of bone marrow cells from a patient with FAB M4 AML. Gates have been placed on two cell populations apparent in the SSC/CD45 plot, one in the usual position of blast cells (orange, R1) and one in a position where monoblasts may be found (blue, R2). 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, human leucocyte antigen (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. (With thanks to Mr Ricardo Morilla.)

Fig. 2.5 (Continued)

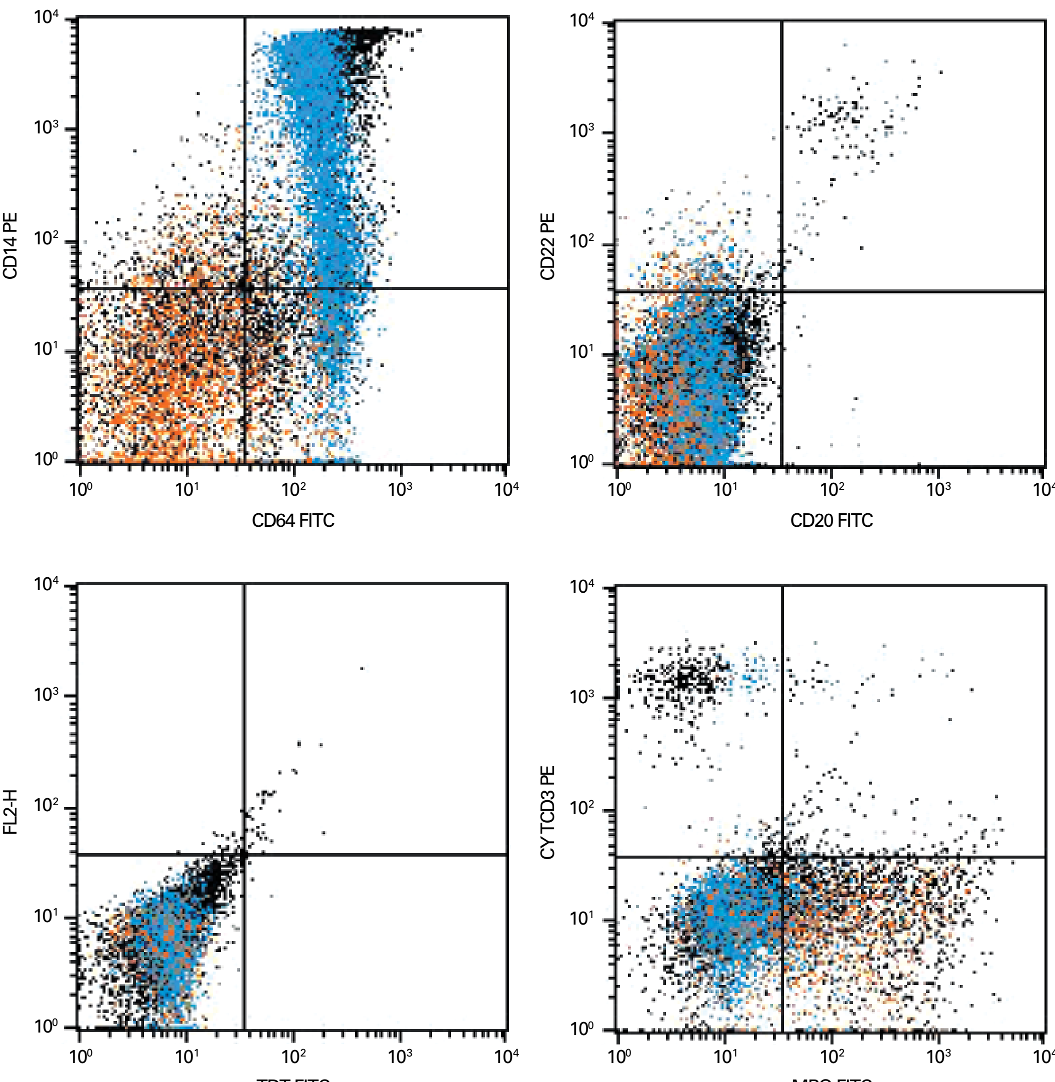


Fig. 2.5 (Continued)

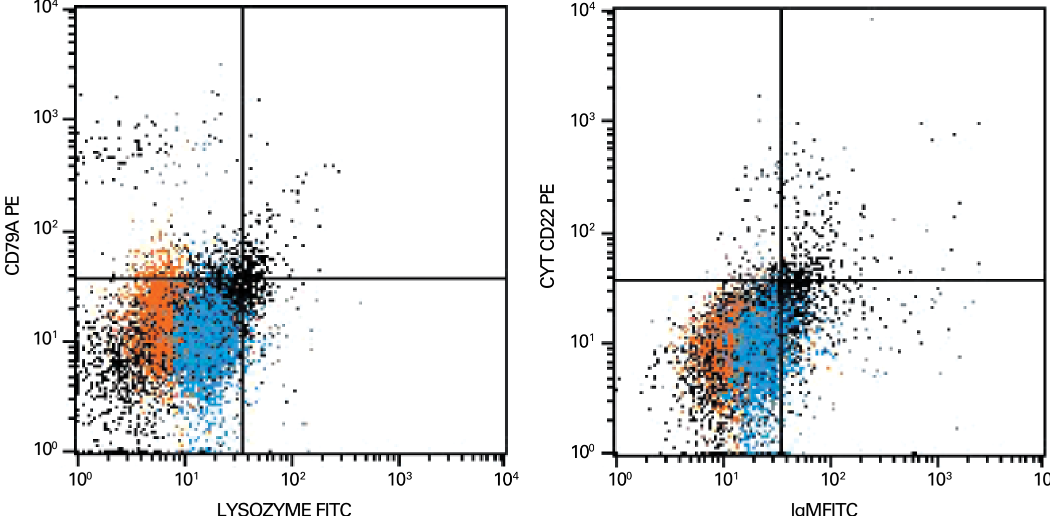


Fig. 2.5 (Continued)

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, for example 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 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. Strength of expression can be indicated by the mean fluorescence intensity, but it is also important to note heterogeneity or homogeneity of expression and the presence of two populations that differ in strength of expression. Comparison of the intensity of expression can be made with an isotype control – that is, an antibody of the same isotype from the same species of animal directed at an irrelevant antigen – or with an internal negative control – 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 (+), 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

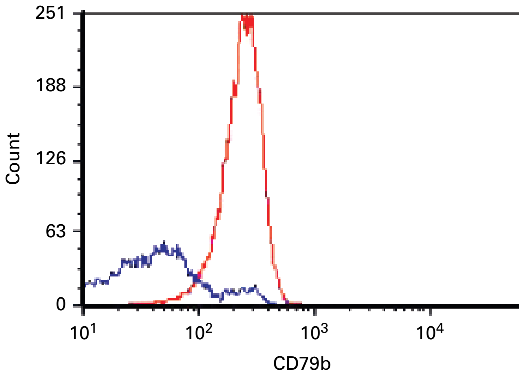


Fig. 2.6 Flow cytometry histogram showing the number of signals plotted against the intensity of the fluorescence signal at the selected wavelength for CD79b (red) and a negative control (blue). These are two populations of cells in the same tube captured in different gates, red representing CD5+ CD19+ 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. (With thanks to Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

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. The presence of dead or dying cells leads to non-specific binding. Non-specific binding can also be due to expression of Fc receptors, for example by cells of monocyte lineage; this can be prevented by pre-incubation with immune sera to block the receptors. Samples anticoagulated with ethylenediaminetetra-acetic acid (EDTA) are generally satisfactory, although light-scattering characteristics of cells are more stable if the sample is taken into preservative-free heparin. Use of EDTA has the advantage that a film for microscopy can be prepared for evaluation in parallel with the results of flow cytometry.

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 are expressed 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. The date and time of venepuncture should be provided.

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; (iv) detecting an aberrant immunophenotype that can be used for monitoring MRD; and (v) monitoring MRD. 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 French–American–British (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 (previously known as 'biphenotypic leukaemia' and 'bilineal leukaemia') and undifferentiated stem cell leukaemia (see below). The immunophenotype can form the sole basis of a classification of AML [8] 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 MRD has become more important, for example 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. The usual approach is to select a panel based on the provisional diagnosis, for example acute leukaemia or a chronic lymphoproliferative disorder, and then investigate further antigen expression selectively.

When flow cytometry is adapted, using an immunobead technique applied to cell lysates, the recurrent cytogenetic abnormalities that can be recognized from the presence of a specific fusion protein include t(15;17)(q24.1;q21.2),

inv(16)(p13.1q22), t(8;21)(q22;q22.1), t(9;22)(q34.1;q11.2), t(1;19)(q23;p13,3) and t(12;21)(p13.2;q22.1) [9].

Problems and pitfalls in immunophenotyping

A bone marrow aspirate may show no immunophenotypic abnormality despite bone marrow infiltration being present, 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, for example in follicular lymphoma or systemic mastocytosis. Similarly, and for the same reasons, flow cytometry immunophenotyping can underestimate the disease burden, for example in multiple myeloma.

Light chain restriction is usually taken as evidence of neoplasia but it should be noted that this is not always so; for example it has been observed in florid reactive follicular hyperplasia [10]. Absent or aberrant expression of antigens can suggest neoplasia but this is not confined to haematological neoplasms. An abnormal T-cell phenotype is not specific for T-cell neoplasia, absence or downregulation of some antigens (including CD7) being seen, for example, in infectious mononucleosis, reactive dermatoses and inflammatory conditions [11]. Neutrophils may fail to express CD16 and CD33 because of a genetic polymorphism and may show aberrant expression of CD64 in sepsis [6]. Myeloid precursor may show aberrant expression of CD56 following administration of granulocyte colony-stimulating factor [6].

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 [12].

Immunophenotyping can only be interpreted with a full knowledge of the ranges of antigen expression by normal cells.

Immunophenotype of normal haemopoietic cells

Normal haemopoietic stem cells express CD34, CD133 and human leucocyte antigen (HLA)-DR. Early neutrophil differentiation is associated with upregulation of CD13 expression and expression of cytoplasmic myeloperoxidase (MPO), followed sequentially by CD15 and CD65 then CD64; at the promyelocyte stage, expression of CD34, HLA-DR and CD117 is lost. Monocytic differentiation is associated with early upregulation of CD64, which is strongly expressed, followed by expression of CD36, CD14, CD35 and then IREM2; expression of CD34 and CD117 is lost whereas HLA-DR expression is retained. CD34-positive haemopoietic progenitors committed to the erythroid lineage first express CD36 then CD105 with loss of CD117, HLA-DR, CD45, CD33 and CD13 at this stage; this is followed by downregulation of CD105 accompanied by strong expression of CD71. The CD34-positive/CD36-positive immunophenotype of early erythroid cells is shared with plasmacytoid dendritic cell precursors, but the latter show strong expression of HLA-DR and strong positivity for CD123. Basophil precursors are also strongly positive for CD123 and in addition express CD203c and lack strong expression of HLA-DR and CD117. Mature basophils express CD9, CD13, CD22, weak CD25, CD33, CD36, CD38 (strong) and CD123 [13]. Mast cell precursors express CD13, CD123 (but more weakly than basophils), usually CD203c and very strong CD117.

The immunophenotype of normal haemopoietic cells during maturation is summarized in Figs 2.7–2.9 [6,14].

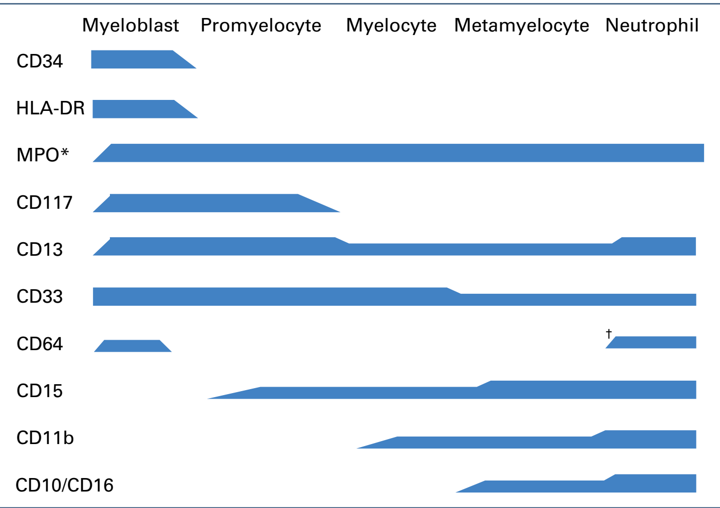


Fig. 2.7 Diagram showing expression of surface membrane and cytoplasmic antigens at various stages of neutrophilic maturation. *MPO expression is cytoplasmic; †CD64 is expressed on neutrophils only when they are activated.

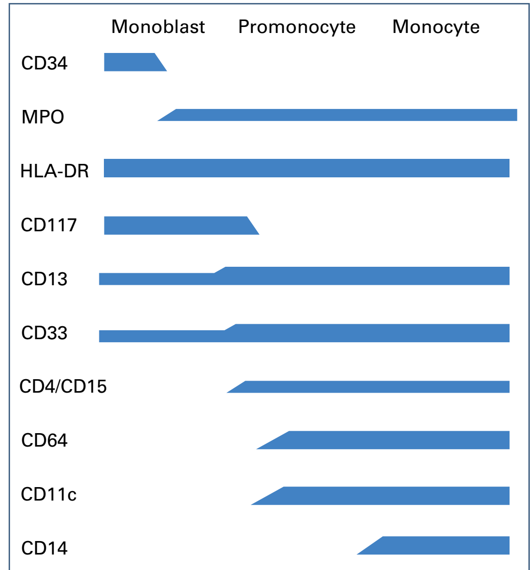


Fig. 2.8 Diagram showing expression of surface membrane antigens at various stages of monocytic maturation.

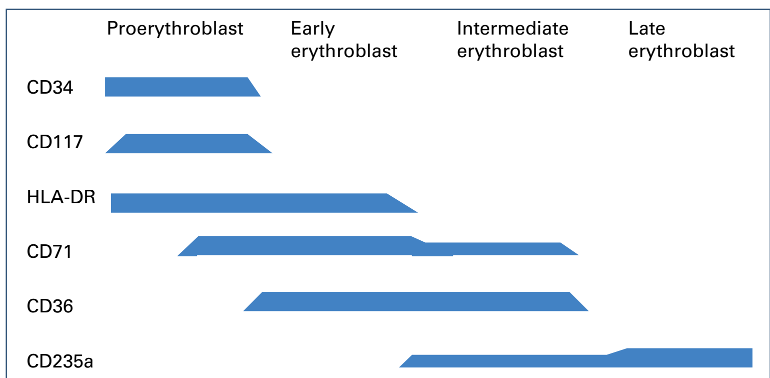


Fig. 2.9 Diagram showing expression of surface membrane antigens at various stages of erythroid maturation.

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 further common indication is for 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 MRD.

Several standard panels for the initial phenotyping of acute leukaemia have been proposed [15–22]. The more important McAb and PcAb used in acute leukaemia diagnosis and classification are shown in Table 2.2 [23], and the antibody panel recommended by the European LeukaemiaNet is in Table 2.3 [24,25]. Similar panels were previously recommended by the European Group for the Immunological Characterization of Leukemias (EGIL) [19], the US–Canadian Consensus Group [21] and the British Committee for Standards in Haematology (BCSH) [22].

As the detection of MRD has become more important in the management of patients with acute leukaemia [26], this role for immunophenotyping has emerged, providing an alternative to genetic analysis for this purpose. A larger panel of antibodies is needed than when immunophenotyping is used only for diagnosis. A large number of events must be analysed, typically 500 000 in comparison with 10 000 for diagnosis, thus permitting detection of 1 abnormal cell in 10 000. It can be useful to have up to four spine antibodies (i.e. antibodies used in every tube), for example CD19, CD10, CD34 and CD45, for MRD monitoring in B-lineage ALL. The leukaemia-associated phenotype may be aberrant (Figs 2.10 and 2.11), very uncommon among normal haemopoietic and lymphoid cells, or not normally found in the bone marrow. Two approaches are possible: a leukaemia-associated phenotype for each individual patient can be identified at diagnosis or leukaemic cells can be identified by their difference from normal cells. 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 [27,28]. 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, that is expression of an antigen inappropriate to the lineage; (ii) under- or overexpression of an antigen, for example absence or downregulation of CD45 or lack of expression of CD13, CD33 or HLA-DR by myeloid cells; (iii) asynchronous expression, for example coexpression of CD3 with either CD34 or terminal deoxynucleotidyl transferase (TdT) or, in the case of myeloid cells, coexpression of CD4, CD11b, CD14, CD15 or CD65 with CD34, or the presence of CD13−CD33+ or CD33 + CD13− cells; and (iv) expression of an antigen inappropriate to a tissue, for example 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 influences patient management, with treatment being reduced in some children with favourable findings [16,17,29–31] and augmented in others. In ALL, MRD at day 8, at day 29 and at end of consolidation are all independent prognostic indicators [29], as is MRD at days 33 and 78 [32]. MRD is also of prognostic significance in AML [30] although the significance differs between different cytogenetic/molecular genetic subtypes. Monitoring MRD during treatment

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

Cluster of differentiation (CD)	Specificity within haemopoietic lineage or other specificity*
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-ALL; blast cells of <10% of cases of T-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-ALL and of a small minority of cases of T-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-ALL and in almost all AML and T-ALL; use of CD45 permits gating on blast cells, which express CD45 and have low side 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-ALL, more weakly expressed in some T-ALL (*c.* 15–20%), expressed in Burkitt lymphoma, most follicular lymphomas and some multiple myelomas, expressed by neutrophils
CD19	B lymphocytes and B-lymphocyte precursors, blast cells of B-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-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-ALL and as a cytoplasmic antigen in *c.* 98%
CD24	B lymphocytes and precursors, blast cells of B-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-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

Table 2.2 (Continued)

Cluster of differentiation (CD)	Specificity within haemopoietic lineage or other specificity*
Antibodies identifying antigens expressed mainly in T cells	
CD1a	Cortical thymocytes, blast cells of about 20% of T-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-ALL, leukaemias of mature T cells; expressed in about 10% of cases of AML, particularly FAB M3 and M4Eo AML/inv(16); expressed by neoplastic mast cells
CD3	Part of the TCR complex; membrane antigen in late thymocytes and mature T lymphocytes, blast cells of *c.* 25% of T-ALL and in leukaemias of mature T cells, cytoplasmic expression is found in the majority of thymocytes and blast cell of T-ALL
CD4	Cortical thymocytes (coexpressed with CD8), subset of late thymocytes, subset of mature T cells, some leukaemias of mature T cells (see Table 7.16), 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-ALL, small subset of B lymphocytes, some leukaemias and lymphomas of mature B cells and mature T cells (see Tables 7.7 and 7.16)
CD7	Thymocytes, majority of mature T cells, NK cells, blast cells of T-ALL, subset of immature myeloid cells, blast cells of 5–15% of AML, some leukaemias of mature T cells (see Table 7.16)
CD8	Cortical thymocytes (coexpressed with CD4), subset of late thymocytes, subset of mature T cells, some cases of T-ALL, some leukaemias of mature T cells (see Table 7.16)
Anti-TCR αβ	Most circulating T lymphocytes and some T-ALL
Anti-TCR γδ	Small subset of circulating T lymphocytes and blast cells of some T-ALL; most cases of hepatosplenic T-cell lymphoma
Antibodies identifying antigens expressed mainly by 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-ALL with t(4;11) but also some T-ALL
CD16	Neutrophils and NK cells, weakly expressed on monocytes

(Continued)

Table 2.2 (Continued)

Cluster of differentiation (CD)	Specificity within haemopoietic lineage or other specificity*
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 neutrophils; expressed preferentially in AML with monocytic differentiation
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-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
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; B-ALL, B-lineage acute lymphoblastic leukaemia; c, cytoplasmic; CD, cluster of differentiation; FAB, French–American–British (leukaemia classification); Ig, immunoglobulin; NK, natural killer; Sm, surface membrane; T-ALL, T-lineage acute lymphoblastic leukaemia; TCR, T-cell receptor.

* For a complete list of the specificities of monoclonal antibodies assigned to each CD category see reference 23.

Table 2.3 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 (Sm or c), anti-SmIg, CD13, CD33, CD34 CD45 for gating purposes
Panel for sublineage classification and definition of clinical entities
Anti-HLA-DR, CD1a, CD4, CD5, CD8, CD3 (Sm), anti-IgM (c), CD14, CD117, CD56, CD65, CD41 or CD61, erythroid marker such as CD235a or CD36
*For orientation of targeted therapy, depending on the type of leukemia and therapy being considered**
CD20, CD33, CD52, CD22

* CD30, expressed in 38% of T-ALL and 13% of B-ALL [25], is another potential therapeutic target.

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

Adapted from Béné *et al.* 2011 [24].

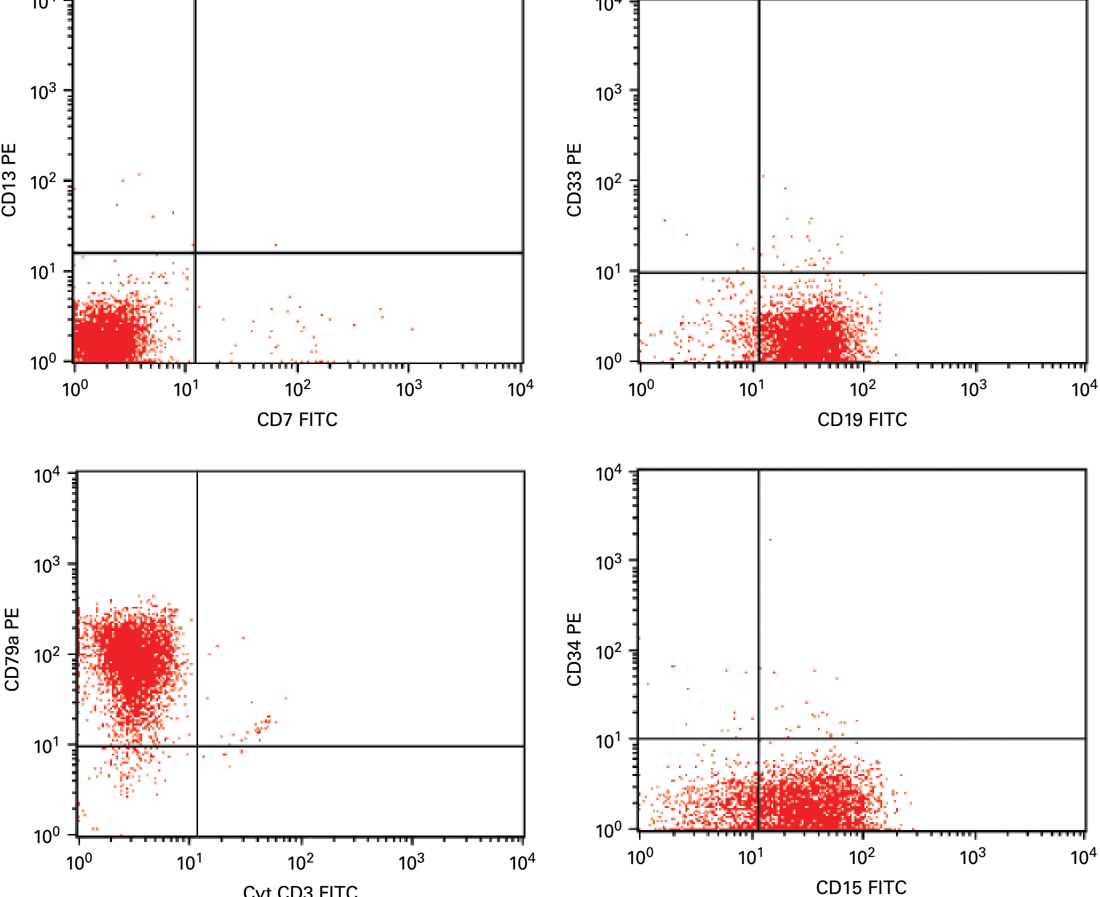


Fig. 2.10 Flow cytometric immunophenotyping in ALL showing aberrant antigen expression that could be used for monitoring minimal residual disease (MRD). The leukaemic B lymphoblasts are appropriately expressing CD19 and CD79a but are also showing aberrant expression of CD15, a myeloid-associated antigen. (With thanks to Mr Ricardo Morilla and Professor Daniel Catovsky, London.)

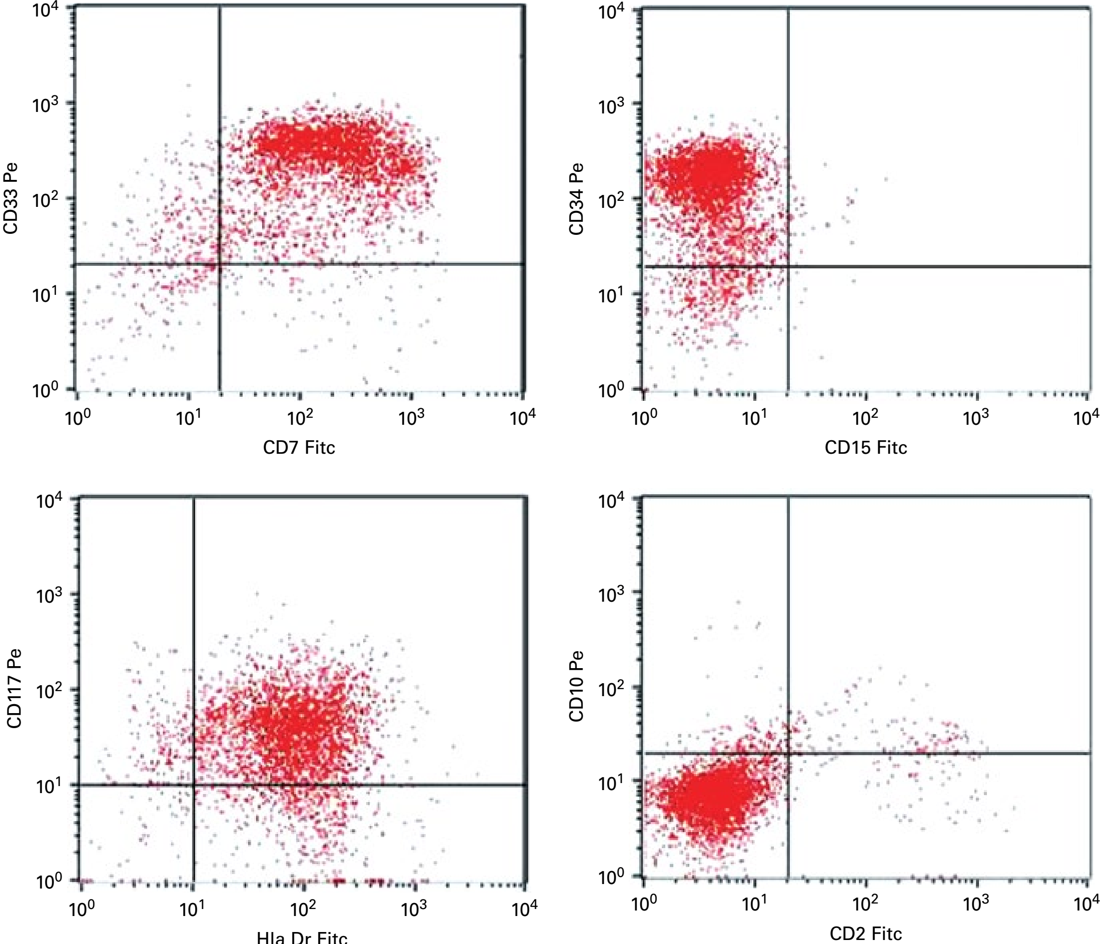


Fig. 2.11 Flow cytometric immunophenotyping in AML showing aberrant antigen expression that could be used for monitoring MRD. There is appropriate expression of CD33 and CD117, which are myeloid-associated antigens, and expression of CD34, a marker of blast cells, but in addition there is aberrant expression of CD7, a T-lymphocyte-associated antigen. (With thanks to Mr Ricardo Morilla and Professor Daniel Catovsky.)

permits an adjusted estimate of the risk of relapse and reduction or augmentation of therapy, depending on the new estimate of risk group [33]. The reappearance of MRD in AML may be an indication for therapeutic intervention, for example for donor leucocyte infusion in a patient who has had a stem cell transplant or for alteration of treatment in acute promyelocytic leukaemia.

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 cytoplasmic 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 [34]. In one study CD13, CD117 and MPO were each expressed in about three-quarters of cases of AML while CD33 was expressed in 88% [34]. CD13 is most sensitive when used with a technique that allows cytoplasmic (c) antigen (cCD13) to be detected, since the antigen appears earlier in the cytoplasm than on the cell membrane [35]. 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 [36], and although a 3% cut-off gives high sensitivity its specificity has not been established [37]. Good sensitivity and specificity is achieved with a cut-off of 13% using an isotype control and 28% using residual lymphocytes as a control [37].

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 [1,20,38–51] (Table 2.4; Fig 2.12). 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 [39,40,48]. Reactions of CD15 antibodies in AML M3 and M5a are less consistent. Positivity for MPO, CD15 and CD65 suggests granulocytic differentiation. Expression of MPO by CD34-positive cells is specific for granulocytic differentiation (CD34 having become negative by the time cells of monocytic lineage express MPO).

CD11b, CD14, CD36 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, M2 and M3 AML [38,40,52]. CD68 is also usually positive in M4 and M5 AML but is only positive in about 40% of other subtypes [44]. 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 [53]. CD87, the urokinase-type plasminogen activator receptor, is also preferentially expressed in M5 AML [54]. 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 [40] 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 (M3V) AML show a characteristic pattern of reaction with McAb, which may be of diagnostic importance in distinguishing M3V from M5b AML (see Table 2.4 and page 150).

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.

Table 2.4 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, 20, 38–51 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; HLA, human leucocyte antigen; TdT, terminal deoxynucleotidyl transferase.
* Also positive in acute lymphoblastic leukaemia (ALL).
† Also positive in B-ALL and in occasional cases of T-ALL.
‡ Also positive in many cases of B-ALL.
§ CD9 positive (note that CD9 is also positive in B-ALL).
** CD36 and CD235a positive.
†† CD9, CD36, CD41, CD42a, CD42b, CD61 positive.

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, strong CD71 reactivity has been considered suggestive of erythroid differentiation since it was rarely seen in other myeloid leukaemias [50]. However, others have found CD71 to be lacking in specificity, being often expressed in AML without maturation [55]. CD36 McAb react also with megakaryoblasts and monocytes [48,56] 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 [57], anti-spectrin and antibodies to the Gerbich red cell antigen. Carbonic anhydrase I, detectable

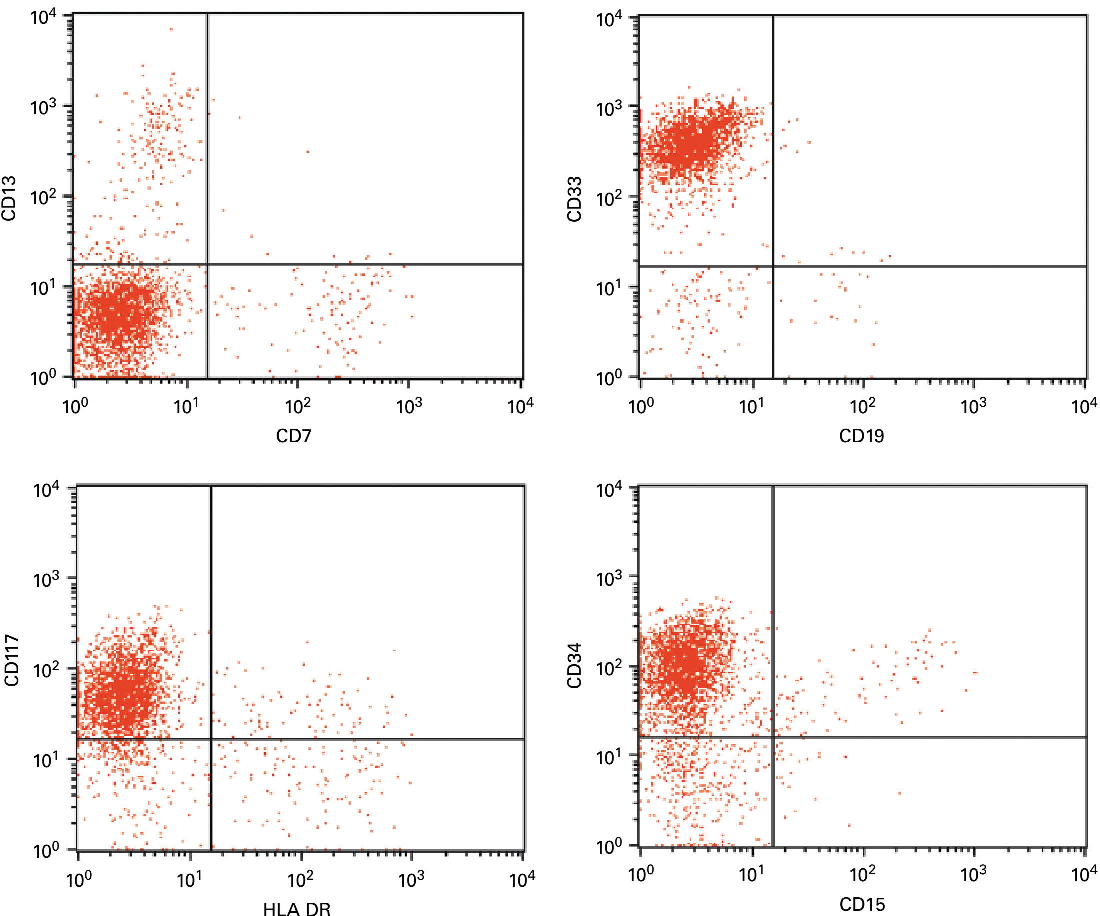


Fig. 2.12 Flow cytometric immunophenotyping in acute promyelocytic leukaemia (FAB M3 AML) showing expression of myeloid-associated antigens, CD33 and CD117, and expression of CD34, a marker of blast cells. There is a failure to express CD13, which is sometimes observed in this subtype of leukaemia, and a very characteristic failure to express HLA-DR. Lack of expression of CD34 is also common. (With thanks to Mr Ricardo Morilla and Professor Daniel Catovsky.)

by a PcAb, is said to be the earliest specific immunophenotypic marker of the erythroid lineage [57].

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 [58]. 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 [59]. 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 [60] (see page 200).

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 [61].

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 [62]; among cases of AML, expression is common in FAB categories characterized by a lack of maturation, that is in M0 and M1 AML. Expression of TdT correlates with expression of CD7 and CD34 [63]. Expression is most common among cases of M0 and M1 AML and in some series also among cases of M2 and M4 AML [38,43,48,64]. 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 [65]; its expression in AML correlates with other markers of immaturity, being most frequent in M0 AML and not being a feature of M3 AML [65].

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 [66]. 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 [64]. 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 [45,67]. 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 [47,68].

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 [69]. Strong CD33 expression has, however, been found to be an independent poor prognostic feature in childhood AML [70]. CD56 expression has been found to correlate with unfavourable cytogenetic abnormalities and with a lower complete remission rate and worse survival [71]. 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;q22.1) and t(15;17)(q24.1;q21.2), respectively [72]. 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) [72]. CD7 positivity in AML has, however, been found not only to correlate with prognostically worse karyotypic abnormalities but also to be indicative of worse prognosis within the group of patients with the most adverse karyotypes [72,73]. CD25 expression is of independent poor prognostic significance in AML [74].

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

Immunophenotyping is usually performed on suspensions of peripheral blood or bone marrow cells but, when necessary, can be carried out, by immunohistochemistry, 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 2.5 [75–77]. Immunohistochemistry can identify an acute leukaemia as myeloid and can identify certain FAB categories, for example 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 [78]. 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.

It should be noted that blast cells should be quantified on stained blood and bone marrow aspirate films, rather than by flow cytometry. Not only may CD34 not be expressed (e.g. by monoblasts) but erythroid precursors may be lost during the cell lysis stage so that the denominator is different [6].

Monitoring minimal residual disease in acute myeloid leukaemia

Immunophenotyping can be used for the detection and quantification of MRD, with a leukaemia-associated immunophenotype being detectable in more than 80% of cases of AML. Early response to treatment can be assessed and has prognostic importance. In one study, the number of cells with a leukaemia-associated immunophenotype at day 16 was found to be prognostically more significant than the percentage of blast cells assessed morphologically [79]. The enumeration of haematogones in first complete remission can also give prognostic information, levels of more than 0.01% being found to be an independent predictor of leukaemia-free survival [80].

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 [81]. 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 [82]. The haemoglobin concentration (Hb) is more often normal in T-lineage disease, an observation confirmed in the same study [82]. The prognostic significance of lineage differs between different series of patients, indicating that prognosis is determined 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 [83]. A higher WBC is indicative of worse

Table 2.5 Monoclonal antibodies and polyclonal antisera useful in the diagnosis of acute leukaemia from decalcified paraffin-embedded trephine biopsy specimens [75–77] (for a larger range of antibodies see reference 77).

Category	Specificity
CD45	Leucocyte common antigen: strong reactions in most lymphoid cells (T and B lineage), weak reactions in blasts of myeloid lineage
Anti-TdT	Positive in lymphoblasts but negative in mature lymphoid cells, positive in blasts in a minority of cases of AML
CD34	Haemopoietic and lymphoid precursors, endothelial cells
CD79a	Positive in B-lineage lymphoblasts and lymphocytes
CD10	Common and pre-B ALL and some Burkitt lymphoma; also positive in follicular lymphoma
CD20	Positive in B-lineage lymphocytes, some B-lineage lymphoblasts and follicular dendritic cells
CD3	Positive in T-lineage lymphoblasts and lymphocytes
Anti-MPO	Positive in blasts in AML except in FAB M7 AML and some cases of M0 AML
Anti-neutrophil elastase	Maturing cells of granulocyte lineage
CD14	Positive in blasts in some cases of AML, mainly FAB M4 and M5
CD15	Positive in blasts in some cases of AML and in Reed–Sternberg cells and mononuclear Hodgkin cells
CD68	Broad specificity; 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)
CD68R	Monocyte restricted
CD117	Haemopoietic progenitors including some proerythroblasts and promyelocytes, blast cells of many cases of AML, mast cells (strongly)
Anti-calprotectin (previously calgranulin)	Positive in most FAB M4 and M5 AML; positive with both granulocytic and monocyte lineages
Anti-lysozyme	Positive in granulocyte and monocyte lineages
CD61	Megakaryocytes and blasts of FAB M7 AML
CD42b	Megakaryocytes and blasts of FAB M7 AML
Anti-von Willebrand factor	Megakaryocytes and blasts of FAB M7 AML
CD235a (glycophorin A)	Erythroid cells
CD236R (glycophorin C)	Erythroid cells

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

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 [83]. 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 [84]. CD79b is expressed later in development than CD79a and is thus less useful. CD10 is usually positive, defining 'common ALL' if cytoplasmic μ chain is not expressed; it is more strongly expressed in B-lineage blast cells than in T-lineage. CD24 is usually positive. CD20 is sometimes positive. CD45 is negative in 15–30% of cases [6]. 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% [34]. It should be noted that, in the WHO classification, cases showing expression of surface membrane immunoglobulin (SmIg) are generally categorized as NHL 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 [85]. When immunohistochemistry is applied, antibodies to CD79a, CD20, CD22, CD10 and PAX5 are useful; PAX5 is more lineage specific, CD79a expression being seen in some T lymphoblasts [86]. However, PAX5 can also be expressed in AML with t(8;21) [86]. CD20 expression was found to have an adverse prognostic significance in adults with Ph-negative B-ALL in one series of patients [87] but not in another [88]. Aberrant expression of myeloid antigens (CD13, CD14, CD15, CD33 or CD65) is not uncommon [6]. Less often there is aberrant expression of T-lineage antigens (CD4 or CD56) [6]. Expression of T-lineage antigens in B-ALL may have an adverse prognostic significance [89].

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 [90]. 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 [91]. 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-ALL and B-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 2.6 [17] and 2.7 [19]. It should be noted that HLA-DR is expressed in the great majority of cases of B-ALL, regardless of the maturity of the cell, whereas among cases of T-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 [92]. 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) and *KMT2A* (previously *MLL*) rearrangement and have a poor prognosis whether or not *KMT2A* is rearranged [93]. Among T-lineage cases, the precise immunophenotype appears to be of less significance.

Table 2.6 European Group for the Immunological Characterization of Leukemias (EGIL) classification of B-lineage acute lymphoblastic leukaemia.

(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.
Adapted from Béné *et al*. 1995 [19].

Table 2.7 European Group for the Immunological Characterization of Leukemias (EGIL) classification of T-lineage acute lymphoblastic leukaemia.

(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.
* It should be noted that many case of pro-T or pre-T ALL meet the criteria for the WHO provisional entity now designated 'early T-cell precursor ALL'.
Adapted from Béné *et al*. 1995 [19].

Classifications of B-lineage ALL reflect a putative normal sequence of B-cell maturation in which 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 [94]. 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 [92]. 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 also in Burkitt lymphoma and follicular lymphoma). Cases with expression of both CD10 and SmIg are generally not ALL, rather representing NHL. Expression of cytoplasmic κ or λ chain also suggests NHL rather than ALL. 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;p13.3), 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 [95]. 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, with regard to complete remission rate, duration of remission and overall survival [96]; in children evidence is conflicting. The adverse prognosis in adults is converted into a better prognosis if rituximab is included in the treatment [97]. High expression of CD40 has been found to correlate with better relapse-free survival in B-lineage ALL on multivariate analysis [98]. 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 lessened.

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 [99]. 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 [100]. 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 [101]. CD81 expression can also be useful, since in comparison with haematogones it is underexpressed in 80% of cases of B-ALL [102]. Approaching 70% of late haematogones express CD5 (which is also expressed on the majority of mature B cells in the bone marrow) [103]. Haematogones can be regarded as the normal counterpart of a leukaemic lymphoblast, and aberrant expression of antigens in leukaemic cells can then be recognized. The differences between haematogones and leukaemic lymphoblasts are summarized in Table 2.8.

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 [104]. 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. Immunophenotyping for CD20, CD22 and aberrant CD33 is relevant to immunotherapy with monoclonal antibodies – rituximab, inotuzumab ozogamicin and gemtuzumab ozogamicin, respectively, having these specificities [105].

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 [15] and in others the categories are increased to four, as in the EGIL classification [19] (see Table 2.7). Cases of T-lymphoblastic lymphoma show a spectrum of maturity from early T-cell immunophenotype to mature thymocyte, but tend on the whole to have a more mature immunophenotype than

Table 2.8 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, CD38 and CD81more strongly expressed	Overall, TdT and CD38 more weakly expressed; CD81 expressed more weakly in 80% of cases
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.

T-ALL [106]. 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 [34]. Myeloid antigen expression (CD13, CD33, CD117) is seen in about 40% of patients and is not of prognostic significance [107]. CD10 is expressed, more weakly than in B-ALL, in about a third of cases. There may also be aberrant expression of CD56 and CD79a.

An early T-cell precursor phenotype, equivalent to a subpopulation of thymocytes that retain multi-lineage differentiation potential, is recognized. Such cases typically show no expression of CD1a or CD8, weak or absent expression of CD5, expression of CD34 and HLA-DR, and expression of CD56 or myeloid markers such as CD11b, CD13, CD33, CD65 and CD117 [108–110]; in comparison with other T-ALL, there is reduced expression of CD2, CD3 and CD4. This early T-cell precursor phenotype is prognostically adverse with regard to event-free survival and overall survival [109].

The various categories of T-ALL have been found to show some prognostic differences but these are less marked than in the case of B-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 [111]. 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 [112]. However, in a study in adults, CD10 was not of prognostic significance [113]. In a German multicentre study in adults, cases classified as pre-T – E-rosette-forming cells (ERFC) negative – had a worse prognosis than T-cell cases (ERFC positive) [114]. 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 [115]. Several studies have shown CD1a expression to be associated with a better prognosis than either a more mature or a less mature immunophenotype [72,116]. 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 [113]. Several studies have expression of TCRγδ to be

associated with a better prognosis than expression of TCRαβ [72]. Patients with T-lymphoblastic lymphoma who have an early T-cell immunophenotype are much less likely than other patients to present with thymic disease [106]. 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, except in the case of early T-cell precursor ALL. 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 [117].

Expression of myeloid antigens is not generally of prognostic significance in ALL [72], 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-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 [113]. A large study in adults with B- or T-ALL, by the same group, found expression of CD13, CD33 or both to be lacking in prognostic significance [118]. However, expression of CD13, observed in half of the patients, was associated with a worse prognosis in a large prospective trial of T-ALL in adults [116].

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 stoichiometrically to DNA. The DNA index compares the amount of DNA with the amount in the nuclei of normal gender-matched cells, such as 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 [33]. Quantitating DNA can also detect multiploidy, that is 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.

Monitoring minimal residual disease in acute lymphoblastic leukaemia

Detection of MRD in ALL by immunophenotyping techniques is about one log less sensitive than detection by molecular analysis. If immunophenotyping is to be used for this purpose, it is necessary to use an appropriate panel of antibodies to recognize a leukaemia-associated immunophenotype [119]. Characteristics sought may be asynchronous expression of antigens (coexpression of markers that are normally expressed on mature and immature cells respectively), aberrant expression of an antigen, inappropriately weak or strong expression of an antigen, 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 [120]. Such expression is uncommon at presentation of B-ALL but at relapse it is significantly more common [120]. In general, at least three or four 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 2.9. Detection of MRD by flow cytometry is less sensitive in B-ALL than T-ALL because of possible confusion of leukaemic blast cells with increased normal B-cell precursors.

Immunophenotyping in mixed phenotype acute leukaemia

Cases of acute leukaemia previously designated as either biphenotypic or bilineage are recognized; acute biphenotypic leukaemia designated cases in which leukaemic cells simultaneously expressed markers of two lineages, usually lymphoid and myeloid, whereas acute bilineage or bilineal leukaemia designated those in which there were two distinct populations of cells of different lineages. The distinction was to some

Table 2.9 Typical antibody combinations for the identification of a leukaemia-related immunophenotype that can be used for detection of minimal residual disease.

Antibody or antibody combination	Abnormality detected
B-lineage ALL	
TdT or CD34 and B-lineage marker coexpressed with CD13, CD15, CD33, CD65, CD66c, CD123 or 7.1/NG.2	Aberrant expression of a myeloid antigen
CD19 plus CD10 coexpressed with CD13, CD15, CD33, CD65, CD66c, CD123 or 7.1/NG.2*	Aberrant expression of a myeloid antigen
TdT or CD34 plus CD10 or CD19 coexpressed with CD56	Aberrant expression of a natural killer/myeloid antigen
TdT or 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
CD45, CD38 or CD81	Underexpression
CD10, CD34, CD58 or CD138	Overexpression
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 expressed on thymic cells (also coexpressed in occasional mature T-cell neoplasms)
CD13, CD33, CD56 coexpressed with a T-cell marker such as CD5	Aberrant expression
CD38 or CD99	Overexpression
AML	
CD33	Overexpression
CD34 or TdT coexpressed with CD11b, CD14, CD15, strong CD33, CD56 or CD65	Asynchronous expression
CD117 coexpressed with CD11b or CD15	Asynchronous expression
HLA-DR and CD15 coexpressed	Asynchronous expression
CD13+CD33–	Lack of synchronous expression
CD13–CD33+	Lack of synchronous expression
CD13–CD15+	Lack of synchronous 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; HLA, human leucocyte antigen; TdT, terminal deoxynucleotidyl transferase.

* 7.1/NG2 is a monoclonal antibody recognizing chondroitin sulphate.

extent artificial, and in the WHO classification both groups of cases are designated 'mixed phenotype acute leukaemia' [121]. The diagnostic criteria are summarized in Table 4.2 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 myelodysplastic syndromes, myeloproliferative neoplasms and overlap syndromes

Immunophenotyping has a role in demonstrating dysplastic maturation in MDS and in characterizing blast populations in MDS and in transformation of myeloproliferative neoplasms (MPN) and the overlap myelodysplastic/myeloproliferative neoplasms (MDS/MPN).

Immunophenotyping in myelodysplastic syndromes

Evidence of dysplastic maturation in MDS may be provided by demonstration of lack of expression of expected antigens (e.g. lack of CD16 or CD33 on neutrophils), asynchronous expression of antigens (e.g. CD34 expression on maturing cells), aberrant expression of antigens (e.g. expression of CD64 on neutrophils or CD56 on myeloid precursors) and reduced SSC by hypogranular neutrophils and precursors. Such evidence is particularly useful when clear morphological signs of dysplasia are lacking. Quantification of CD34-positive cells can also be helpful in making a distinction between hypoplastic MDS and aplastic anaemia, 1% or more CD34-positive cells being indicative of MDS [122]. Blast cells in MDS may have immunophenotypic abnormalities but if the blast count is increased this is not usually of any importance in diagnosis. Myeloblasts are not infrequently MPO negative. They may show asynchronous expression of CD10, CD11b and CD15 and aberrant expression of lymphoid antigens such as CD4, CD7 and CD56.

Immunophenotyping in myelodysplastic/myeloproliferative neoplasms

Dysplastic features similar to those in MDS may be demonstrable in MDS/MPN but immunophenotyping is rarely needed for diagnosis of these conditions.

Immunophenotyping in myeloproliferative neoplasms

Immunophenotyping does not have any role in the diagnosis of chronic phase MPN but can be used to characterize blast cells following transformation.

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 locus (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 monoclonal antibody 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. Use of both polyclonal and monoclonal antibodies has been advised to increase the detection rate of surface membrane light chain, particularly in CLL when expression is weak [123].

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 7.

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; (iii) uniform expression of CD26 on a T-cell population (this antigen normally being expressed only on a proportion of T cells); or (iv) 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) [124]; 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. A further 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) and in certain congenital immune deficiencies (CD4−CD8−).

As for the B lineage, expression of CD34 or TdT excludes a diagnosis of T-NHL (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-NHL 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 downregulated 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 7.

Immunophenotyping in suspected natural killer (NK)-lineage lymphoproliferative disorders

NK 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 PcAb and some McAb 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, fluorescence *in situ* hybridization (FISH), 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. If bone marrow aspiration fails it may be possible to carry out cytogenetic analysis on peripheral blood cells, if the blast cell count is high, or it may be possible to obtain cells by agitation of a trephine biopsy specimen in a suitable medium. 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. 2.13). 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 2.10 [125]. 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 [125]. Translocations are written as follows: t(15;17)(q24.1;q21.2), which indicates that there is a reciprocal translocation between chromosomes 15 and 17; the

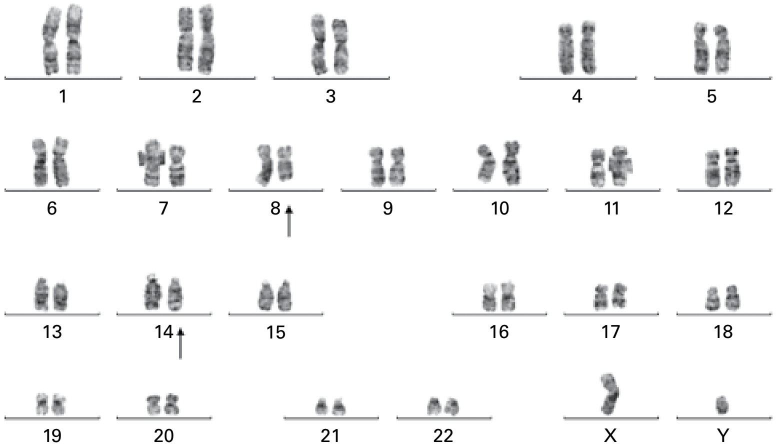


Fig. 2.13 Karyogram from a patient with Burkitt lymphoma showing chromosomes identified by Giemsa banding. There is t(8;14)(q24;q32) with the part of chromosome 8 that is translocated being longer than the part of 14 that is reciprocally translocated; as a result there is shortening of the long arm of 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. (With thanks to Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

Table 2.10 Abbreviations, terminology and symbols used in describing chromosomes and their abnormalities.

add	Additional material of unknown origin
aneuploid	Cells having an abnormal number of chromosomes 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)(q34.1;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)

(Continued)

Table 2.10 (Continued)

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
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
rob	Robertsonian translocation, a whole long arm translocation between two acrocentric chromosomes with loss of the short arms
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, surround the number of cells
,	Comma, separates number of chromosomes, sex chromosomes and chromosome abnormalities, e.g. 46,XY,inv(3)(q21.3q26.2)
.	Decimal point, separates band number from sub-band number
;	Semicolon, separates chromosomes and breakpoints in structural rearrangements

Table 2.10 (Continued)

+	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)(q34.1;q11.2)[6]//46, XY[14] indicates six recipient metaphases in a patient with chronic myeloid leukaemia and 14 donor metaphases

breakpoints are at band q24 sub-band 1on chromosome 15 and at band q21 sub-band 2 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 necessarily indicate a neoplastic condition; for example loss of the Y chromosome is more often an age-related phenomenon. In one study it was identified in 10.7% of patients with MDS, 4% of patients with AML and 7.7% of patients without a haematological neoplasm [126]. In a second study −Y was present in a higher percentage of metaphases in patients with a haematological neoplasm, and its presence in >75% of metaphases was predictive of a neoplastic condition [127]. Similarly, loss of Y in 100% of metaphases is more common in AML than in patients without a haematological neoplasm [126]. Clonal abnormalities sometimes occur without any apparent haematological disorder, for example being observed, rarely, in donor cells following haemopoietic stem cell transplantation [128]. 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 – as instanced by t(12;21)(p13.2;q22.1) in ALL. Other rearrangements, such as 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. It is not rare for a significant chromosomal rearrangement to be missed on cytogenetic analysis. In one study in which conventional cytogenetic analysis was compared with molecular analysis for seven common recurrent rearrangements in patients with acute leukaemia, 7/57 cases were missed (most having a normal karyotype) representing a false negative rate of 12%; in addition, cytogenetic analysis failed in 9% of 362 cases [129]. However, it should be noted that a molecular technique such as multiplex polymerase chain reaction (PCR) will detect only the specific genetic abnormalities being sought, so that cytogenetic and molecular analysis should be regarded as complementary.

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 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 [130] (Table 2.11). 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;q22.1) could be recorded as .nuc ish (ETO × 2),(RUNX1 × 2)(ETO con RUNX1 × 1), 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. 2.14). Split signal FISH is very useful when a single oncogene has multiple partners, for example the *KMT2A* gene in acute 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. 2.15). 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.

Table 2.11 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
×	Multiplication sign, precedes number of signals, e.g. −×2 indicates signals missing on the two homologous chromosomes
.	Full stop, separates cytogenetic results from *in situ* hybridization results
;	Semicolon, separates probes on different derivative chromosomes, e.g. 46,XX,t(9;22)(q34.1;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) × 2[100] indicates that 100 interphase nuclei have been assessed and all have the normal number of BCR and ABL1 signals nuc ish(ABL1 × 2),(BCR × 2),(ABL1 con BCR × 1)[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

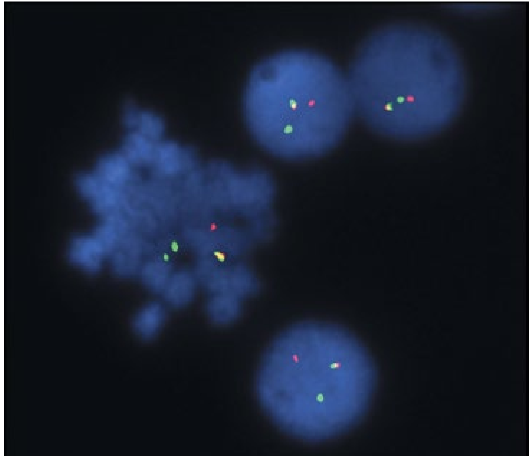


Fig. 2.14 Fluorescence *in situ* hybridization (FISH) using a dual-colour, break-apart probe for *RARA*. As a result of t(15;17)(q24.1;q21.2) in a patient with acute promyelocytic leukaemia the *RARA* gene has been split. Normal cells would have two red-yellow-green fusion signals whereas these leukaemic cells have one normal fusion signal and separate red and green signals, on derivative chromosomes 17 and 15 respectively. (With thanks to Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

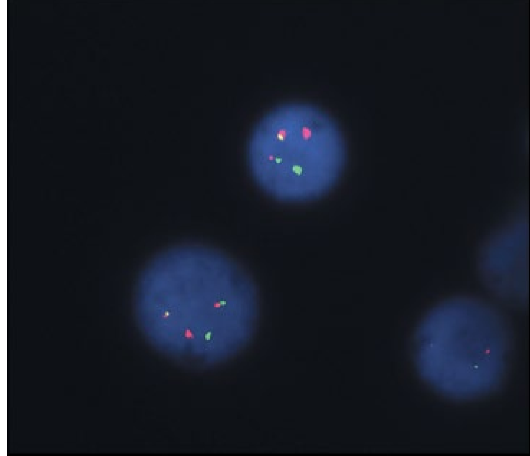


Fig. 2.15 FISH using a dual-colour, dual-fusion technique. Normal cells would have two red signals (*PML* on chromosome 15) and two green signals (*RARA* on chromosome 17). As a result of t(15;17)(q24.1;q21.2) in a patient with acute promyelocytic leukaemia, one allele of each gene has been split with 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. (With thanks to Dr Helen Wordsworth and the staff of Sullivan Nicolaides Pathology, Brisbane.)

Kits are available that permit multiple FISH hybridizations (e.g. 5–10) spatially separated on a single slide. The panel can be selected to cover B- ALL, T-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. 2.16).

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 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; for example analysis for *BCR-ABL1* fusion in imatinib-treated chronic myeloid 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

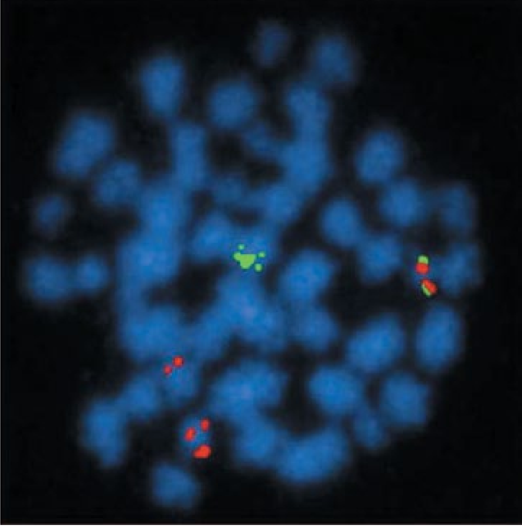


Fig. 2.16 FISH of chromosomes of a patient with ALL and the common translocation, t(12;21)(p13.2;q22.1), associated with a complex karyotype: (a) metaphase by multicolour FISH; (b) the same metaphase hybridized with probes to *ETV6-RUNX1* (green-red) showing the gene fusion (red and green together), 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. (With thanks to the Leukaemia Research Cytogenetics Group, Newcastle University, Newcastle upon Tyne.)

Table 2.12 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 *KMT2A* that 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

(*Continued*)

Table 2.12 (Continued)

Technique	Principle	Application
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
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 TP53) or that a protein product has an abnormal distribution (e.g. PML protein in acute promyelocytic leukaemia and ALK in anaplastic large cell lymphoma)

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 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 2.12. 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).

Molecular genetic analysis can also be based on microarray analysis, in which the patient's fluorescently labelled DNA or RNA is bound to an array of complementary DNA or oligonucleotides on a glass slide. By comparison with a reference DNA, unbalanced DNA aberrations can be detected. RNA analysis can be specifically for protein-coding RNA (gene expression profiling) or for non-coding RNA, such as microRNA. Such analysis is based on single nucleotide polymorphisms and is thus often referred to as SNP analysis. Gene expression profiling by microarray analysis is likely to become increasingly important in the analysis of leukaemic cells, both for research and, in clinical practice, 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. SNP analysis permits the detection of acquired copy-neutral loss of heterozygosity, a major mechanism by which both copies of a tumour suppressor gene are

inactivated or by which oncogenic mutations become homozygous.

Next generation gene sequencing can be employed to analyse the whole genome, the whole exome or the whole transcriptome, or a specific panel relevant to a disease can be employed, for example to detect cancer-predisposing mutations or to detect mutation of genes known to be associated with myeloid neoplasms. Single base substitutions can be recognized. The sensitivity is 3–5% of neoplastic cells.

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 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 2.13 [131,132].

Table 2.13 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?) [131], familial t(3;6)(p14;p11) [132]
Recognition of subtypes of AML with differing prognosis requiring differing therapeutic approaches, including confirmation of acute promyelocytic leukaemia by demonstration of t(15;17)(q24.1;q21.2)
Confirmation of AML rather than MDS in patients with a low blast percentage, e.g. in patients with t(8;21)(q22;q22.1) and inv(16)(p13.1q22)
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 (good), t(9;22)(q34.1;q11.2) and t(4;11)(q21.3;q23.3) (bad)
Confirmation of a diagnosis of chronic myeloid leukaemia by demonstration of t(9;22)(q34.1;q11.2)
Confirmation of a diagnosis of chronic eosinophilic leukaemia by identification of a clonal abnormality
Identification of chronic eosinophilic leukaemia or other myeloid or lymphoid neoplasm associated with rearrangement of *PDGFRB* by demonstration of a clonal rearrangement with a 5q32 breakpoint
Identification of chronic eosinophilic leukaemia or other myeloid or lymphoid 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)(q13.3;q32) or a variant translocation
Confirmation of a diagnosis of follicular lymphoma by demonstration of t(14;18)(q32;q21.3) or a variant translocation
Confirmation of a diagnosis of Burkitt lymphoma by demonstration of t(8;14)(q24.2;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

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

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 [133], 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 [134].

Further information can be derived from molecular genetic analysis (Table 2.14). The purpose of molecular genetic analysis may be either the establishment of clonality, by detection of rearrangement of either an IGH or TCR 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 NHL with such high specificity that the subtype may be defined more accurately by the karyotype than by the morphology.

Table 2.14 Some examples demonstrating the role of molecular genetic analysis in haematological neoplasms.

Recognition of subtypes of AML with differing prognoses 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 biallelic *CEBPA* mutation (good prognosis)
Confirmation of AML rather than MDS in patients with a low blast percentage, e.g. in patients with *RUNX1-RUNXT1* or *CBFB-MYH11* fusion genes
Recognition of therapy-related AML, e.g. by demonstration of *KMT2A* 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)(p13.2;q22.1)
Recognition of prognostically significant cytogenetic subgroups in patients with B-ALL and failed or normal cytogenetic analysis
Confirmation of a diagnosis of Ph-positive ALL or chronic myeloid leukaemia by demonstration of *BCR-ABL1*
Confirmation of a diagnosis of a myeloid or lymphoid neoplasm associated with rearrangement of *PDGFRA* by demonstration of *FIP1L1-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/s.

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. 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 CLL 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 [135]. The cytogenetic and molecular genetic techniques now available include: (i) standard cytogenetic analysis; (ii) FISH; (iii) PCR; (iv) RT-PCR; (v) RQ-PCR; and (vi) gene expression profiling by microarray analysis. It could be speculated that in the future the diagnosis of AML and of B- and T-ALL might be made by microarray analysis [136,137].

Minimal residual disease in acute leukaemia

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.

Monitoring minimal residual disease and adapting treatment can improve the outcome in patients with acute promyelocytic leukaemia with t(15;17)(q24.1;q21.2) and acute myeloid leukaemia with t(8;21)(q22;q22.1) [138].

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 [139].

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 this study as having at least three numerical or structural abnormalities) show an almost 30-fold increase [140]. 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 [141]. 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 [142].

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 generally indicative of a considerably worse prognosis [143]. Overall, this poor prognosis is not improved by transplantation [144]. *FLT3* mutations are less common in children than in adults, being found in only 10–15% of paediatric patients [145]. Further molecular indicators of worse prognosis include partial tandem duplication of *KMT2A* (*KMT2A*-PTD),

WT1 mutation, overexpression of *EVI1* (which may indicate cryptic chromosomal rearrangement) and overexpression of *BAALC*, *MN1*, *ERG* or *WT1*.

Molecular genetic analysis has been used to demonstrate the intrauterine origin of some cases of AML associated with t(8;21) [146].

The technique of microarray analysis is becoming increasingly important in AML. The gene expression pattern differs between AML subtypes, for example AML associated with *RUNX1-RUNX1T1*, *PML-RARA*, *CBFB-MYH11*, *KMT2A* 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 [147–150]. Microarray analysis can be used to identify expression of prognosis-associated genes in paediatric [151] and adult [149,152] 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 at least two types of mutation, a class I mutation that leads to a proliferation/survival advantage and a class II mutation that interferes with differentiation [153]. However, there may be some fusion genes that are in themselves sufficient to cause the leukaemic phenotype, such *FUS-ERG*, *KMT2A-MLLT1*, *KMT2A-MLLT3* and *MYST3-NCOA2* [154]. Mathematical modelling also suggests that an *NPM1* mutation may be sufficient in itself to cause leukaemia [155]. 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. It is also possible to recognize two further types of mutation contributing to the development of haematological neoplasms, specifically type III mutations (in tumour suppressor genes such as *WT1* and *TP53*) and type IV mutations, in epigenetic regulator genes – influencing DNA methylation or chromatin structure, such as mutations in *IDH1*, *IDH2*, *TET2*, *AXSL1*, *DNMT3A* and *EZH2*. Clonal haemopoiesis with mutations in genes including *ASXL1*, *DNMT3A* and *TET2* is found in a proportion of elderly individuals and is predictive of haematological neoplasia [156]. Some of these non-random associations are shown in Table 2.15 [157–159]. 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 [130,160], many of which are recurrent (i.e. they have been reported in more than one patient, often occurring in a significant proportion of patients) [161]. 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 less often occur in secondary leukaemia, and erythroid and megakaryocyte dysplasia is 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;q34.1), t(1;7)(q10;p10), t(3;3)(q21.3;q26.2)

Table 2.15 Class I and class II mutations that can interact in the pathogenesis of acute myeloid leukaemia [157–159].

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;q22.1)	*KIT* mutation (12–47% of cases)	Worse [157,158]
	NRAS (*c.* 10%)	No difference [159]
	FLT3-ITD (*c.* 4%)	
CBFB-MYH11 resulting from inv(16)(p13.1q22) or t(16;16)(p13.1;q22)	*NRAS* (*c.* 30-40%)	No difference [159]
	FLT3-ITD (*c.* 7%)	
	KIT mutation (22–47% of cases)	No difference [157] or worse [158]
PML-RARA resulting from t(15;17)(q24.1;q22.1)	*FLT3*-ITD (*c.* 30%)	Possibly worse
	NRAS (*c.* 2%)	
CEBPA mutated	*FLT3*-ITD	Worse
NPM1 mutated	*FLT3*-ITD	Worse

ITD, internal tandem duplication.

* Reported incidence in subtype shown in brackets. *BCR-ABL1* and *PTPN11, JAK2, SOCS1* and *SOCS3* mutations are also type I.

and inv(3)(q21.3q26.2) occur in multiple morphological subtypes and in MDS as well as in both *de novo* and therapy (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, MPN or MDS/MPN. 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 although the prognostic ranking has not been identical in different series of patients (Table 2.16) [160,162–172]. 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 [166]. 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 were therefore reassigned to the intermediate prognosis

Table 2.16 Prognostic significance of haematological, cytogenetic and molecular genetic abnormalities in acute myeloid leukaemia [160,162–172].

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;q22.1) t(15;17)(q24.1;q21.2) 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 excluding t(3;5)(q21~25;q31~35), inv(3)(q21.3q26.2), t(3;3)(q21.3;q26.2), t(10;11)(p13;q23.3), abnormal 17p plus other abnormality, −17, abnormal 17p with other changes, t(6;11)(q27;q23.3), t(10;11)(p11~13;q23.3), t(9;22)(q34.1;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 autosomies or monosomy of one autosome plus a structural abnormality – 'monosomal karyotype'
Molecular	*NPM1* mutated (in the absence of *FLT3*-ITD) Biallelic *CEBPA* mutation *IDH2* mutated		*FLT3*-ITD Mutation of *WT1** *KMT2A*-PTD* Overexpression of *BAALC**, *ERG**, *WT1*, *EVI1**, *MN1**, *CD34** *KIT* mutation – in cases with t(8;21) or t/inv(16) *ERG* mutated

ITD, internal tandem duplication; PTD, partial tandem duplication; WBC, white blood cell count.
* In patients with normal cytogenetic analysis.

group. This prognostic categorization now determines treatment selection in UK trials. A group of patients with autosomal monosomies (see Table 2.16) were found to have a highly adverse prognosis, and if they were removed from the complex karyotype group the significance of this category was not so adverse [167,170]. A later study found that −5/5q− and abnormalities of 17p were adverse after haemopoietic stem cell transplantation, and that if this was allowed for, a monosomal karyotype and complex cytogenetic rearrangements lost their significance [169]. However a further study did not confirm this [173].

Molecular genetic abnormalities also have independent prognostic significance (see Table 2.16). Analysis for *CEBPA* biallelic mutations, *IDH2* mutation and high expression of CD34 is sufficient to define different prognostic groups within the cytogenetic intermediate prognosis group [172]. Genetic analysis for three fusion genes and for mutations in six genes can divide cases of AML into five prognostic groups [174], the prognostic information provided being stronger than that yielded from cytogenetic analysis.

The prognostic significance of cytogenetic and molecular genetic abnormalities in AML is discussed further in Chapter 3.

Cytogenetic and molecular genetic analysis in acute lymphoblastic leukaemia

Genetic abnormalities in ALL include the primary abnormality, often detectable on cytogenetic analysis, and secondary abnormalities, either gene mutation or loss, that are preferentially associated with specific primary abnormalities [175]. The latter include genes encoding transcription factors or involved in lymphoid differentiation, cell cycle regulation, proliferation or cell survival [175].

Cytogenetic analysis

The role of cytogenetic analysis in ALL is similar to its role in AML [176]. In addition, it can be important in distinguishing Burkitt lymphoma from cases of B-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)(p13.2;q22.1) (*ETV6-RUNX1* being detected by molecular means). Worst prognosis is associated with t(9;22), *KMT2A* rearrangement and hypodiploidy (fewer than 44 chromosomes).

Occasionally cytogenetic analysis has yielded new information on leukaemogenesis, indicating the intrauterine origin of some cases of childhood B-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 *TRD-LMO2* fusion [177].

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)(p13.2;q22.1).

In infants and children, molecular genetic analysis has been of value in determining whether leukaemia has its origin in intra- or extra-uterine life. Using such techniques to demonstrate specific fusion genes, comparison of cord blood samples with samples taken after the development of leukaemia has 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 [146]. 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-ALL. Specifically, deletion of one of these genes, *IKZF1*, is often associated with *BCR-ABL1*-positive ALL and *BCR-ABL1*-like ALL and has been linked to a poor prognosis [178,179]. With gene expression profiling it is possible to recognize characteristic gene expression signatures associated with T-ALL and with B-ALL subtypes – t(1;19) (*TCF3-PBX1*), t(12;21) (*ETV6-RUNX1*), t(9;22) (*BCR-ABL1*), t(8;14) (*MYC* translocated), *KMT2A* rearrangement, high hyperdiploidy and 'other B-lineage', although cases with *BCR-ABL1* fusion show heterogeneity [137,148,178,180,181]. Furthermore a distinction can be made between three different translocations that lead to *KMT2A* rearrangement – t(4;11), t(9;11) and t(11;19) – and between cases with t(4;11) with *HOXA* gene expression and without *HOXA* expression (high risk of relapse) [182]. 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 [183]. Microarray analysis can also permit recognition of clusters of cases that differ from, and overlap with, known cytogenetic/molecular genetic categories but that may nevertheless have biological significance. Thus two clusters can be recognized within T-ALL and seven clusters within B-ALL, which do not relate closely to cytogenetic subtypes [184]. Gene expression in T-ALL differs from that in T-lymphoblastic lymphoma [181].

An early T-cell precursor phenotype can be recognized from its gene expression profile and has a bad prognosis [178]. A *BCR-ABL1*-like group has been recognized from its characteristic gene expression signature [178]. This novel group of childhood ALL represents about 15% of cases of ALL and has been associated with a poor prognosis [179]. *IKZF1* deletion occurs in 10–15% of cases of ALL and in about 40% of cases with a *BCR-ABL1*-like signature and is also prognostically adverse [179]. Drug-resistant ALL can be distinguished from fully sensitive ALL with a correlation with treatment outcome being observed [185]. 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 [150]. Gene expression profiling can demonstrate inactivation of the tumour suppressor gene, *CDKN2A*, associated particularly with *BCR-ABL1*- and *TCF3-PBX1*-positive cases [181].

The prognostic significance of cytogenetic and molecular genetic abnormalities in ALL and mixed phenotype acute leukaemia is discussed further in Chapter 4.

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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3

Acute Myeloid Leukaemia: Integration of Morphological, Immunophenotypic and Genetic Information and the WHO Classification

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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 and its 2016 revision 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 2016 revision of 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, 2001 and 2008. 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 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. Patients with 20–29% bone marrow blast cells do not necessarily have the same disease characteristics as patients with ≥30% blast cells; they are likely to be older, have more high-risk cytogenetic abnormalities and are less likely to have *NPM1* and *FLT3*-internal tandem duplication (ITD) mutations [5]. In those aged 70 years or older, survival may be similar to that of patients with MDS with <20% blast cells and better than that of patients with ≥30% blast cells, with hypomethylating agents being at least as effective as intensive chemotherapy [5].

The WHO classification of AML is summarized in Table 3.1 [6]. Classification is hierarchical, as shown in Fig. 3.1, with therapy-related cases being assigned first, then cases with certain specified recurrent genetic abnormalities,

Table 3.1 The 2016 revision of the WHO classification of acute myeloid leukaemia (AML).

Therapy-related myeloid neoplasms
AML with recurrent genetic abnormalities*
AML with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*
AML with inv(16)(p13.1q22) or t(16;16)(p13;q22); *CBFB-MYH11*
AML with t(15;17)(q24.1;q21.2); *PML-RARA*
AML with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*
AML with t(6;9)(p23;q34.1); *DEK-NUP214*
AML with inv(3)(q21q26.2) or t(3;3)(q21;q26.2); *GATA2*, *MECOM*
AML with t(1;22)(p13.3;q13.1); *RBM15-MKL1*
AML with mutated *NPM1*
AML with biallelic mutated *CEBPA*
Provisional entity: AML with *RUNX1* mutation
Provisional entity: AML with t(9;22)(q34.1;q11.2); *BCR-ABL1*
AML with myelodysplasia-related changes
AML not otherwise specified
AML with minimal differentiation
AML without maturation
AML with maturation†
Acute myelomonocytic leukaemia
Acute monoblastic/monocytic leukaemia
Pure erythroid leukaemia
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.

† 'with granulocytic maturation' is intended

Adapted from Arber *et al.* 2017 [6].

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.

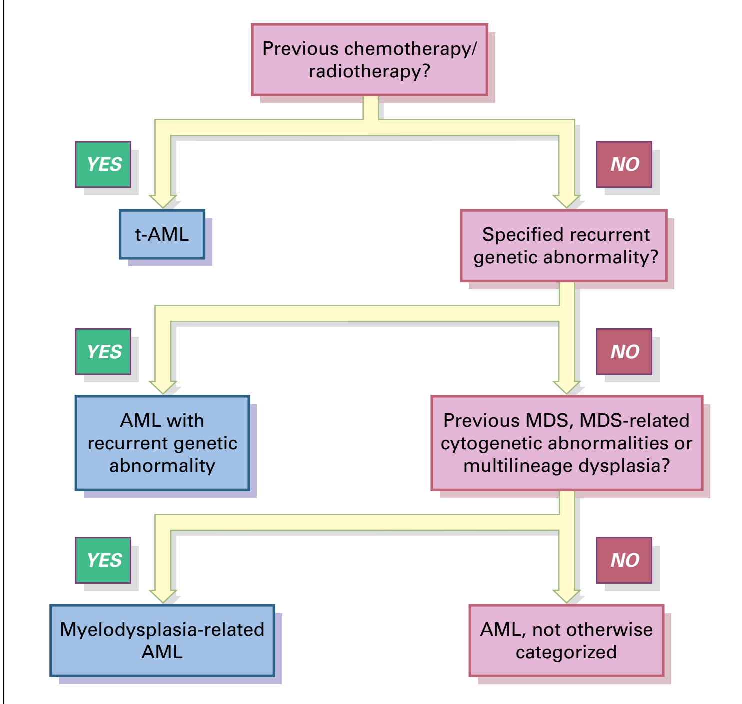


Fig. 3.1 Algorithm showing how the 2016 revision of the World Health Organization (WHO) classification of acute myeloid leukaemia (AML) is applied; Down syndrome-associated myeloid neoplasms and myeloid sarcoma are not included. MDS, myelodysplastic syndrome.

Acute myeloid leukaemia with recurrent genetic abnormalities

In the 2016 revision of the WHO classification 'AML with recurrent genetic abnormalities' now includes eight cytogenetic/molecular categories, one of them provisional, and a further three molecularly defined categories, one of them provisional [7]. In addition, 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.

Acute myeloid leukaemia with t(8;21)(q22;q22.1); *RUNX1-RUNX1T1*

t(8;21)(q22;q22.1) [8–13] (Fig. 3.2), reported by Rowley in 1973 [8], 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 [14–16]; in a series of 1897 unselected adult patients prevalence was 4.6% [17]. The frequency is higher in children (12–14% of AML) than in adults (6% of AML) [15,18]. In elderly adults, the prevalence falls to 2% [19]. 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* [20]; a second mutation occurring in extrauterine 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 [11] and in China [21]. The prevalence of t(8;21) is similar among *de novo* and therapy-related cases, but the latter

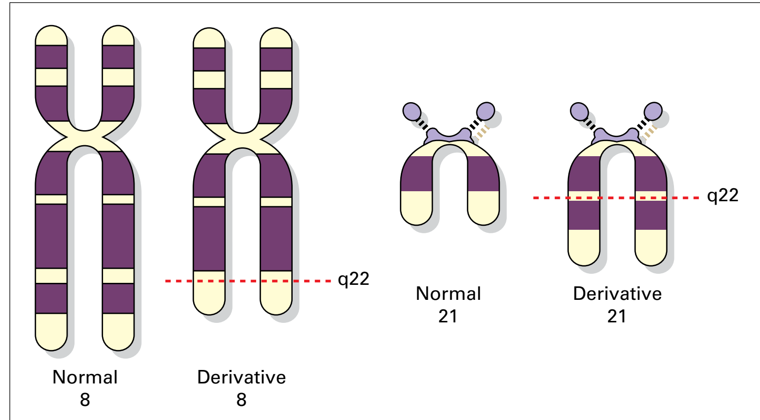


Fig. 3.2 A diagrammatic representation of the t(8;21)(q22;q22.1) abnormality. The breakpoints in the two derivative chromosomes are indicated. Modified from reference 2.

are assigned to the category of therapy-related AML (t-AML) [17]. Therapy-related cases generally follow exposure to topoisomerase II-interactive drugs including etoposide and the anthracyclines [22,23]; they have a significantly worse prognosis, with a median survival of 19 months in one series of 13 patients [24] and a 5-year survival of only 30% [25]. There may be a preceding myelodysplastic phase and trilineage myelodysplasia in t-AML with t(8;21). This subtype of leukaemia may also be linked to prior exposure to benzene [25]. There appears to be a more than coincidental association between this subtype of AML and systemic mastocytosis [26].

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. Formation of myeloid sarcomas, solid tumours of leukaemic cells, is not uncommon [27] and may be the presenting feature, with the bone marrow and peripheral blood sometimes being normal [28]. Rarely a leukaemic pleural effusion is present [29].

Characteristic cytological features are observed [12,13,30,31] (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 nucleoli. Cytoplasm may be basophilic (Fig. 3.3b), sometimes vacuolated and often with a paranuclear paler hof. 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 [9]. Neutrophils often show hypogranularity, bizarre-shaped nuclei and the acquired Pelger–Huët anomaly. Homogeneous salmon-pink cytoplasm of mature neutrophils is characteristic [31]. Maturing granulocytes may contain Auer rods, which are sometimes even found in metamyelocytes and neutrophils (Fig. 3.3a). Auer rods may also be observed in macrophages (Fig. 3.3b). An unusual feature, striking haemophagocytosis by neutrophils, has been described in one patient [32]. 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 [33]; although some may

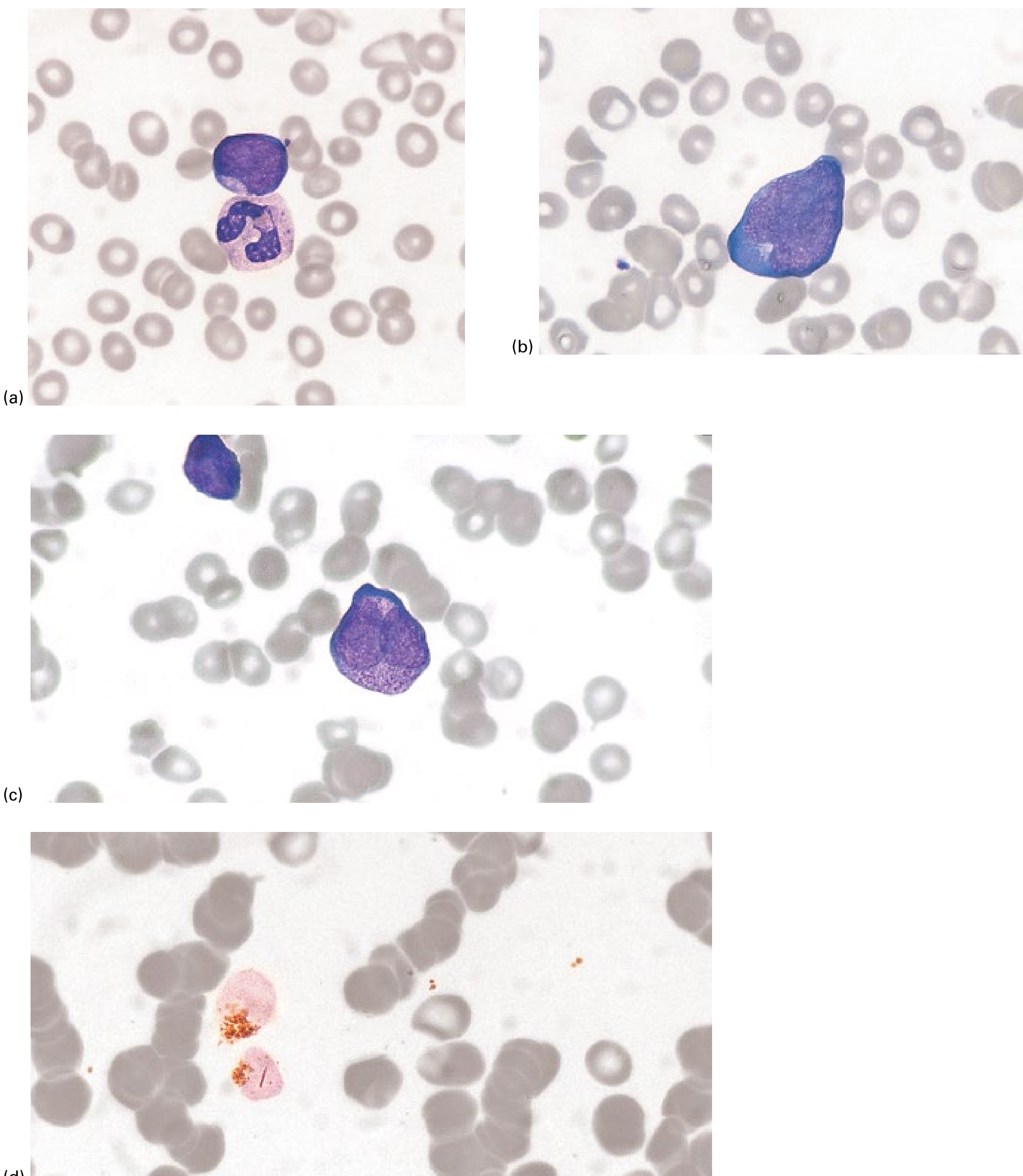


Fig. 3.3 Peripheral blood (PB) and bone marrow (BM) films of a patient with AML associated with t(8;21)(q22;q22.1), French–American–British (FAB) M2 AML. (a) PB film showing a blast cell and an abnormal neutrophil. May–Grünwald–Giemsa (MGG) × 100. (b) PB film showing a blast cell with strongly basophilic cytoplasm and a paranuclear hof representing the Golgi zone. MGG × 100. (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.

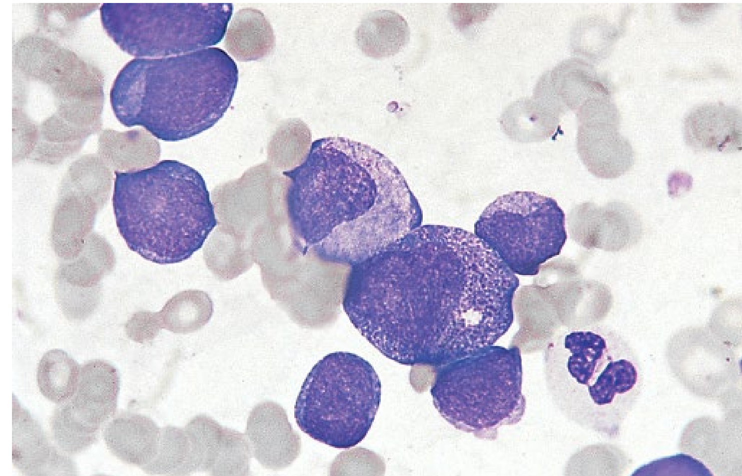
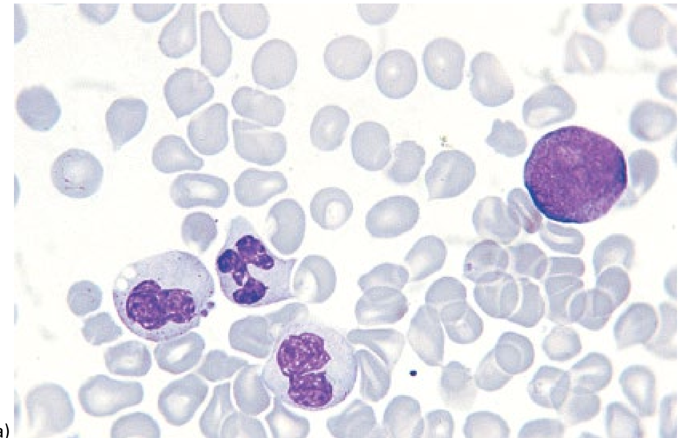
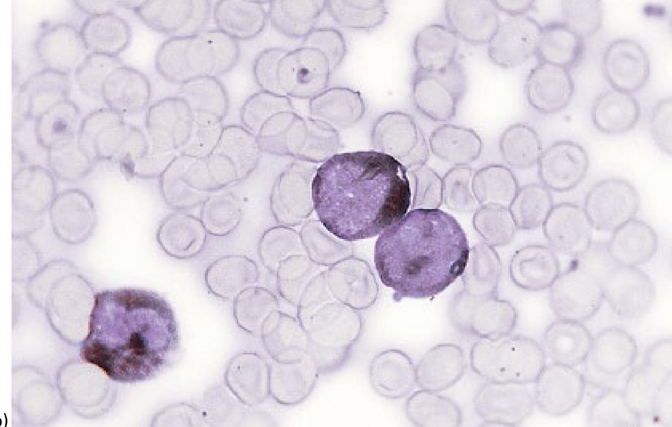


Fig. 3.4 BM film from a patient with AML associated with t(8;21)(q22;q22.1), FAB M2 AML, showing blasts and maturing granulocytic cells including a hypogranular neutrophil. MGG × 100.

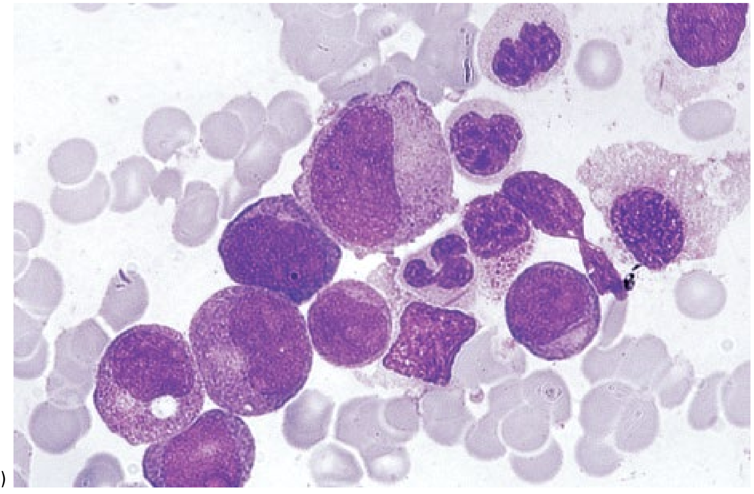


(a)



(b)

Fig. 3.5 BM film of a patient with AML associated with t(8;21)(q22;q22.1), 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.


(a)

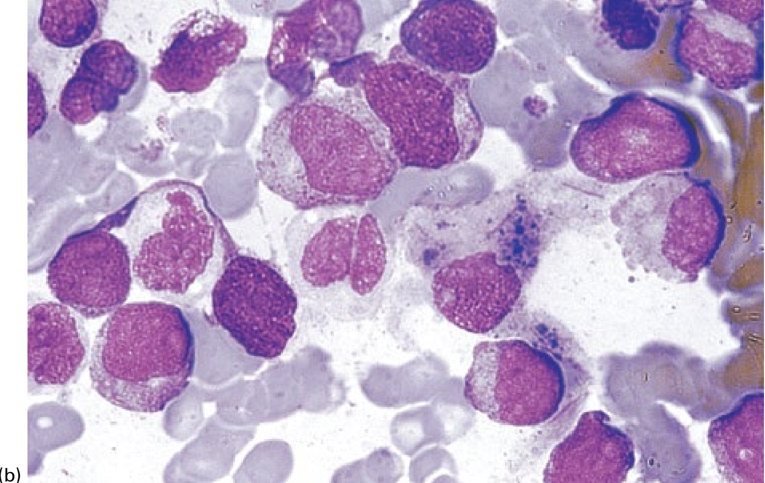

(b)

Fig. 3.6 BM film from a patient with AML associated with t(8;21)(q22;q22.1), 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.

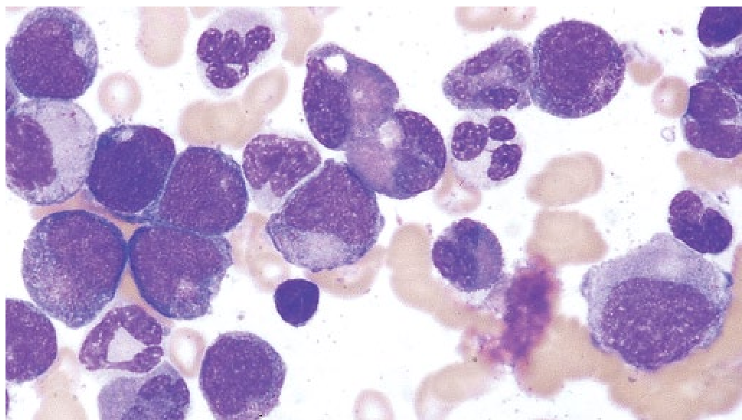


Fig. 3.7 BM film of a patient with t(8;21)(q22;q22.1), FAB M2 AML with eosinophilia, showing maturing cells of neutrophil and eosinophil lineage; there are three eosinophil myelocytes and two mature eosinophils. MGG × 100.

have granules that are basophilic this feature is much less marked than in inv(16)/M4Eo (see below) [10]. Most patients do not have a peripheral blood eosinophilia but occasional patients have had a markedly elevated eosinophil count [34–36], sometimes with an associated hypereosinophilic syndrome [35]. An increase of bone marrow basophils or mast cells occurs in a significant proportion of cases [30] and sometimes this is striking [37]. The mast cells were demonstrated, in one patient, to be part of the neoplastic clone [38]. Rarely the presentation is as acute basophilic leukaemia [39,40]. 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 [13] and not present in others [12,31]. 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 [36,41] and their recognition as AML in the WHO classification is clearly appropriate.

Cytochemical stains [12,42] 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 be Golgi zone positivity for α-naphthyl acetate esterase [13]. 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) [10]. The neutrophil alkaline phosphatase score is generally low [43], but neutrophils that are negative for SBB and MPO are uncommon [42]. 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) [12] (see below).

The complete remission rate and median survival are relatively favourable, with a complete remission rate of around 90% and a reported 5-year survival of 50–70% with chemotherapy alone [19,44–48]. 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% [49]. The prognosis in children appears similarly good [48]. Although the prognosis is good in younger patients, it is intermediate in older patients [50]. Prognosis is significantly better in those with a presenting white blood cell count (WBC) of 20×10^9/l or less [51]. 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 [52]. In view of the favourable prognosis with chemotherapy alone, stem cell transplantation in first remission is considered not to be indicated. In one large series, survival was no better with stem cell transplantation [52]. Intensive treatment with high-dose cytarabine appears to be important in achieving long-term survival [47].

This translocation retains a relatively good prognostic significance in patients in first relapse and is incorporated into the European Prognostic Index [53].

Immunophenotype

This category of AML has a characteristic immunophenotype [54] (Fig. 3.8). Blast cells express CD34, human leucocyte antigen DR (HLA-DR), CD117 and MPO in more than 95% of cases [55]. They are also typically positive for CD13 (75%), CD33 (>85%) and CD65 [55]. Rare cases are negative for CD13, CD33 and CD14 but are positive for MPO [56,57]. 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 [58]. Expression of CD13 is usually,

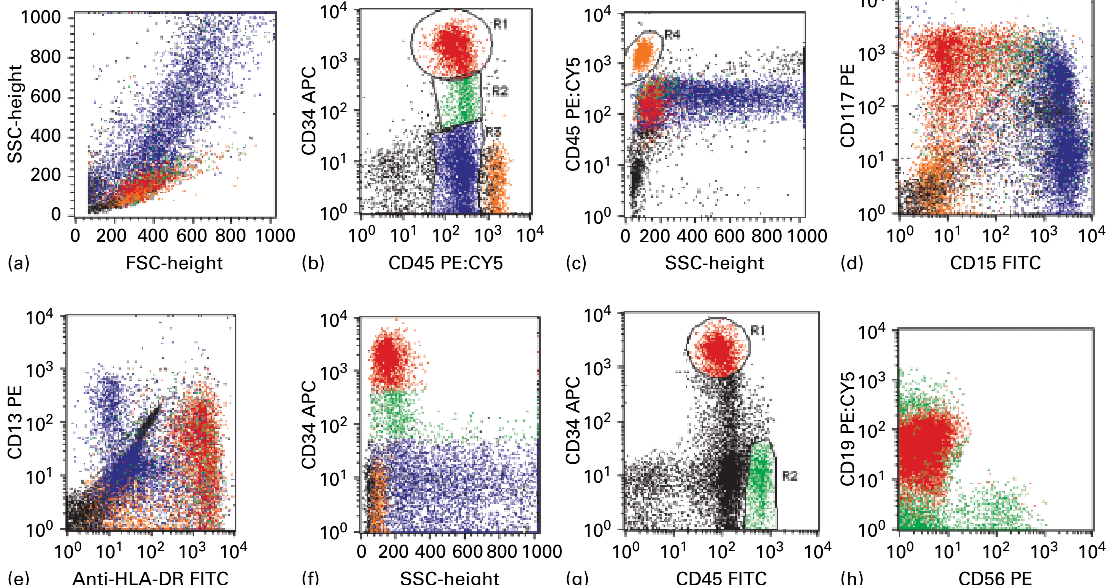


Fig. 3.8 Four-colour flow cytometry immunophenotyping in a patient with AML associated with t(8;21)(q22;q22.1). Plots a, b and c show standard analysis regions set to delineate myeloid blast differentiation (compare with Fig. 2.3); plots d and e show CD117/CD15 and CD13/HLA-DR expression by these individual 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. Plot f shows CD34+ blast cells with low SSC. Plots g and h show CD34 and CD19 expression by blasts cells, though at levels weaker than on normal B cells. (With thanks to Dr Steve Richards, Leeds.)

however, stronger than expression of CD33 [59]. CD11b and CD15 are expressed mainly on the maturing granulocytic cells [60] but aberrant coexpression of CD34 and CD15 is seen in a significant minority of cases. CD11c is expressed in half or more of cases [55,61]; 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 [62]. There is usually positivity for the B-lineage marker CD19 (not very strong; 75% of cases in a large series) [55] and often for the natural killer cell (NK) marker CD56 (also weak; 82% in the same series) [55,60,63,64]. Expression of CD56 correlates with a worse prognosis [65,66]. CD79a is expressed in many cases, 6 of 69 in one study [67] and 10 of 19 in another [55], 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 [68]. Coexpression of CD34, HLA-DR and MPO has approaching 100% sensitivity for detection of this subtype [61]. Coexpression of CD19 and CD34, which is uncommon in other subtypes of AML, suggests a diagnosis of AML with t(8;21) [69], with coexpression of CD19, CD34 and CD56 having about 67% sensitivity and approaching 100% positive predictive value [61]. CD2, CD4, CD7, CD14, CD64 and terminal deoxynucleotidyl transferase (TdT) have been reported to be usually negative [56,60,63] and, when TdT is positive, expression is usually weak [7]; in one study TdT expression was reported more often positive [61]. 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 [70]. The B-cell-associated transcription

factor, OCT2, may be expressed [71]. Expression of CD56 and lack of expression of CD19 has been found to be suggestive of cases with a *KIT* mutation [55].

Cytogenetic and molecular genetic features

The t(8;21)(q22;q22.1) 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), t(8;11;21) and t(8;13;21) associated with *RUNX1-RUNX1T1* fusion, have the same disease characteristics [72,73]. MAC (morphology–antibody–chromosomes) techniques show the translocation in cells of granulocytic lineage but not in erythroid or megakaryocytic cells [74]. Eosinophils are also part of the neoplastic clone [10]. Common secondary karyotypic abnormalities are loss of the Y chromosome in males, loss of the inactive X chromosome in females and del(9q) with loss of 9q22. 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 [45] but, in another series of patients who had either t(8;21) or inv(16), survival was worse in those with complex cytogenetic abnormalities [46]. In one study, loss of a sex chromosome was found to be associated with a worse prognosis [75], and in another −Y was associated with a better prognosis, but neither of these observations was confirmed in other studies [48]. A small study suggested that del(9q) worsened the prognosis but this was not substantiated in a larger study [76]. 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 [49].

The molecular mechanism of leukaemogenesis is fusion of part of the *RUNX1* gene at 8q22 with part of the *RUNX1T1* gene from 21q22.1 [77]. 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 of gene expression, AML associated with t(8;21) can be distinguished from AML with t(15;17) or inv(16)/t(16;16) [78].

FLT3 mutations are considerably less common than in AML in general [79,80]; in one large study *FLT3*-ITD was found in 15% and a *FLT3* tyrosine kinase domain mutation (*FLT3*-TKD) in 7% [81]. Mutations of *KIT* occur in a significant minority of patients – reported in 10% [82], 17% [80] and 45% (including 28%

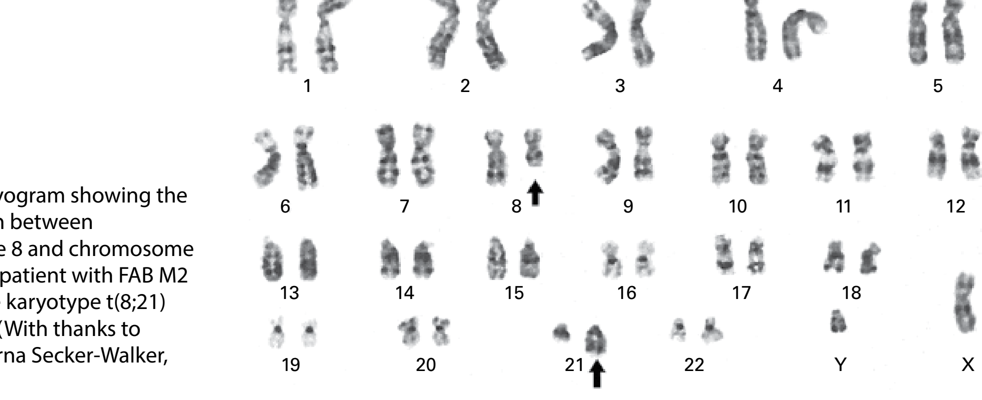


Fig. 3.9 Karyogram showing the translocation between chromosome 8 and chromosome 21 in a male patient with FAB M2 AML and the karyotype t(8;21)(q22;q22.1). (With thanks to Professor Lorna Secker-Walker, London.)

D816 mutations) [83]. *KIT* mutations, or at least *KIT* D816 mutations, are associated with a higher WBC [83], more extramedullary disease [83], a greater risk of relapse [80,83,84] and a worse survival [80,82,83]. 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 [85]. Some of the patients with a *KIT* mutation have aberrant bone marrow mast cells [82,86]. *KIT* mutations are also associated with other translocations that lead to rearrangement of the *RUNX1* gene, for example t(3;21), t(12;21) and t(17;21) [82]. *JAK2* V617F is more common than in AML in general [87]. *RAS* mutations were found to be more common than in AML in general in one study [88] but not in two others [89,90]. Mutations can be in *KRAS* or *NRAS* and do not influence prognosis [90]. Mutations in *ASXL1* are found in about 11% of patients, and in *ASXL2* in approaching a quarter of patients, the two being mutually exclusive; both are associated with a worse prognosis [91].

t(8;21) can be detected using dual-colour, dual-fusion fluorescence *in situ* hybridization (FISH) and probes for *RUNX1* and *RUNX1T1* [92] (Figs 3.10 and 3.11). The rearrangement can also be detected 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 [30,93]. Use of molecular techniques has been reported to increase the number of cases identified by up to 60% in comparison with cytogenetic analysis alone [94], but in another large study only 2 of 33 cases (6%) were not detected by cytogenetic analysis [16].

The detection of minimal residual disease (MRD) does not necessarily presage haematological relapse. However, when MRD is monitored by a real-time quantitative polymerase chain reaction (RQ-PCR), the quantity of fusion

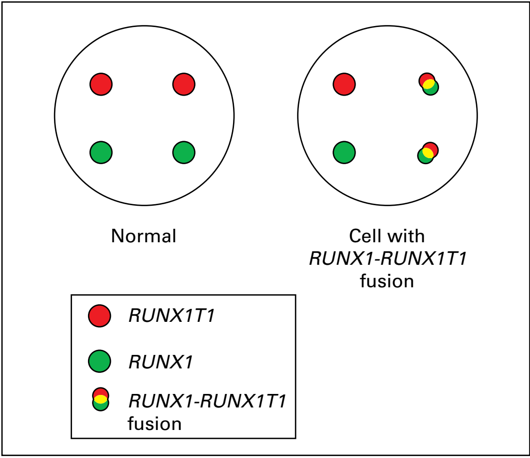


Fig. 3.10 Diagrammatic representation of dual-colour, dual-fusion fluorescence *in situ* hybridization (FISH) for the detection of *RUNX1-RUNX1T1* (*AML1-ETO*). The normal cell has two red *RUNX1T1* (*ETO*) signals and two green *RUNX1* (*AML1*) signals. The cell with a t(8;21) translocation has a normal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

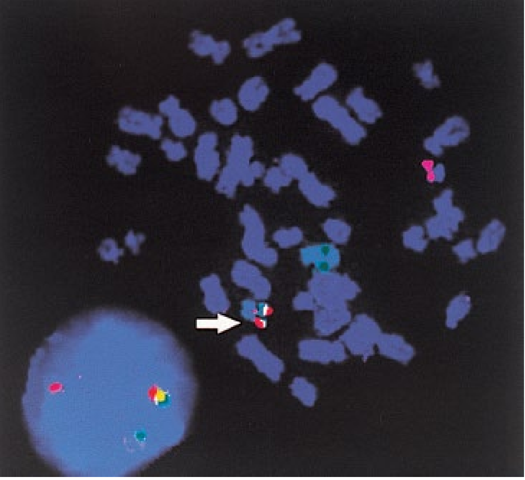


Fig. 3.11 Metaphase spread and interphase nucleus from a patient with FAB M2 AML and t(8;21)(q22;q22.1) hybridized with probes to the genes *RUNX1T1* (*ETO*) (green) and *RUNX1* (*AML1*) (red). These are located at 8q22 and 21q22, respectively. Note one green signal (on the normal 8), one red signal (on the normal 21) and one yellow signal (on the derived chromosome 8) indicating the fusion of the two genes resulting from the translocation. (With thanks to Professor Christine Harrison, Newcastle.)

gene transcript present at presentation and after consolidation chemotherapy is predictive for event-free survival [95]. A 1 log or greater increase in transcript from the remission level is predictive of relapse [96].

Acute myeloid leukaemia with other *RUNX1* rearrangement

Acute myeloid leukaemia associated with t(16;21)(q24.3;q22.1) and *RUNX1-CBFA2T3* (previously known as *AML1-MTG16*) appears to be closely related to AML with t(8;21), with a similar molecular mechanism, usually FAB M2 morphology with or without eosinophilia and possible coexpression of CD19 and CD34 [97,98]. There may be peripheral blood eosinophilia [98]. Cases with t(16;21) are usually but not always therapy related (and then classified as such) and there may be preceding MDS. Trisomy 8 is a frequent secondary abnormality [99]. 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)(q24.1;q21.2); *PML-RARA*

t(15;17)(q24.1;q21.2) (Fig. 3.12), is present in the great majority of patients with acute promyelocytic leukaemia (APL) (FAB types M3 and M3V AML) and, with the exception of rare conditions such as hypergranular promyelocytic transformation of either chronic myeloid 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 [100,101]. The microgranular variant was described in 1980 [102] and the hyperbasophilic microgranular variant in 1982 [103]. An abnormal chromosome 17 was first reported in 1976 [104], and in 1977 the same group recognized the reciprocal translocation between chromosomes 15 and 17 [105]. Overall, this subtype constitutes 4–9% of cases of AML in different series of patients [14,15]; in a series of 1897 unselected adult patients prevalence was 5.2% [17]. Among cases of AML in elderly adults, the prevalence falls to 4% [19]. This type of leukaemia is more common in Costa Rica, Nicaragua and Venezuela, and in Mexican Mestizos and the Hispanic population in the USA [106]. 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 [107]. Prevalence does not differ significantly between *de novo* and therapy-related cases of AML [17], and in one series of patients 10% of cases of APL were therapy related [108], following topoisomerase II-interactive drugs and other agents [22,23,109]. The epidermal growth factor inhibitor, gefitinib, used to treat carcinoma of the lung, appears to be another potential cause [110]. Therapy-related cases, which are classified as such rather than as 'AML with recurrent genetic abnormality',

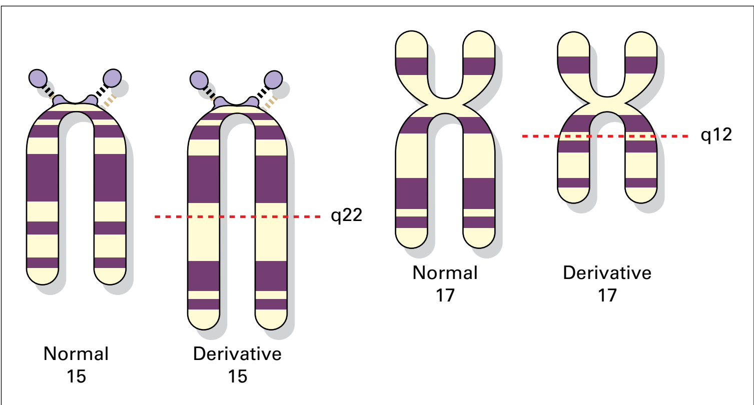


Fig. 3.12 A diagrammatic representation of the t(15;17)(q24.1;q21.2) abnormality. Modified from [2].

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. Therapy-related AML with this translocation is significantly more likely to have secondary chromosomal abnormalities [108].

Clinical and haematological features

Disseminated intravascular coagulation (DIC) with markedly increased fibrinolysis is common, and patients often present with haemorrhage. Thrombosis, particularly cerebral thrombosis, occurs in a minority of patients, 8% in one series [111]. The distinctive cytological and cytochemical features of hypergranular and the variant form of APL, including the hyperbasophilic variant, have been described on page 23 and are illustrated in Figs. 3.13–3.15. Rarely cases are morphologically atypical, for example with FAB M1 features [112]. Rarely there is marked bone marrow fibrosis [113]. Presentation with extramedullary disease is rare [114]. The platelet count tends to be lower than in other types of acute leukaemia. The WBC is often normal or low in hypergranular APL but usually elevated in the microgranular/hypogranular variant, median counts being 1.8 and 15.8×10^9/l respectively in a large series of patients [115]. A WBC of 50×10^9/l or higher is an adverse prognostic feature, but this is negated by treatment with regimes that include all-*trans*-retinoic acid (ATRA)/arsenic trioxide [116]. An uncommon manifestation is haemolytic uraemic syndrome with schistocytes in the blood film and thrombotic microangiopathy affecting the kidneys [117]. Although *de novo* APL does not show dysplasia of other lineages, both erythroid and megakaryocytic dysplasia are quite common in t-AML with the same translocation [108].

Historically, the complete remission rate and median survival were relatively favourable [19,44,45], and in recent years these have improved further. There is a particular sensitivity to differentiation therapy with ATRA, a discovery first made in China by Zhen-Yi Wang and colleagues [118], 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% [48,119,120] or higher. A study of 759 patients entered into MRC trials showed a 10-year survival of 73% [49]. Five-year

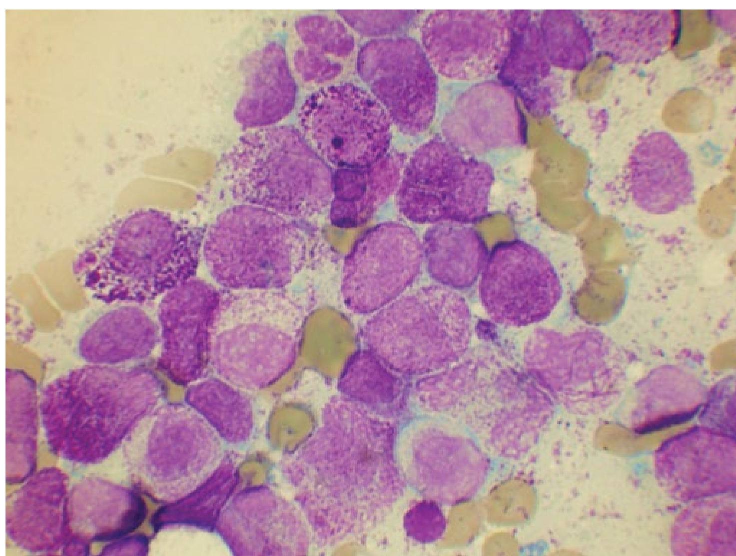


Fig. 3.13 BM aspirate in acute promyelocytic leukaemia showing hypergranular promyelocytes, several with large granules and one containing numerous Auer rods. A bilobed nucleus is apparent in one cell. MGG × 100.

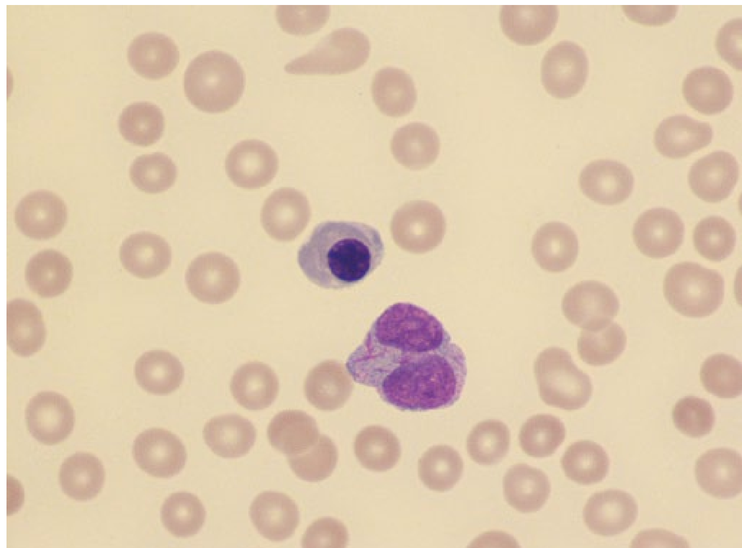


Fig. 3.14 PB film in the variant form of promyelocytic leukaemia showing an erythroblast and a variant promyelocyte with a typical bilobed nucleus; the cytoplasm contains Auer rods. MGG × 100. (With thanks to Dr Wenchee Siow, London.)

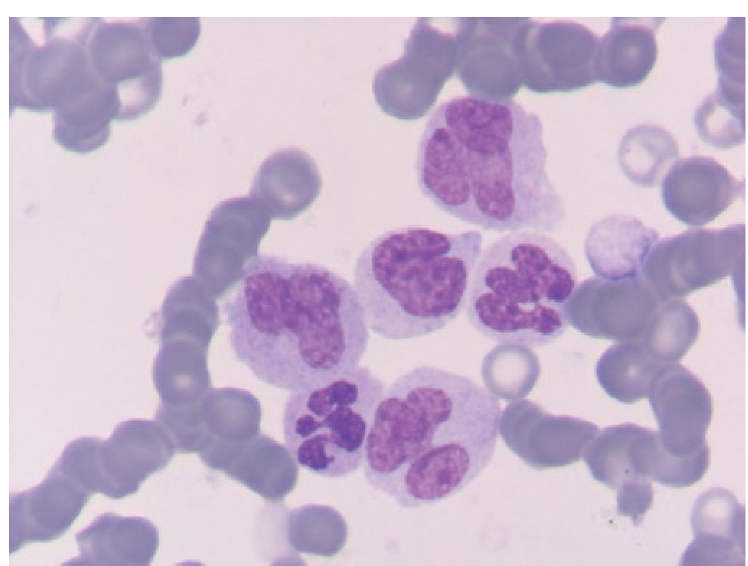


Fig. 3.15 PB film of a patient with acute promyelocytic leukaemia, showing abnormal maturation of leukaemic cells during treatment with all-*trans*-retinoic acid. MGG × 100. (With thanks to Dr Abbas Hashim Abdulsalam, Baghdad.)

survival in t-AML with this translocation approaches 50% and is thus considerably better than in most other types of t-AML [109]. 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 [121]. 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 [121]. Prognosis is best in those with a lower presenting WBC (less than 2, 5 or 10×10^9/l in different trials [120,122–124]) and a presenting platelet count greater than 40×10^9/l [120,125]; 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 [115,124] but not in others [126], but in view of the higher WBC that characterizes the microgranular variant it is likely that there is a genuine difference in prognosis. In a large series of patients, the early death rate was significantly higher in the microgranular variant, the overall survival and disease-free survival were worse, and the cumulative risk of relapse was higher but all differences became non-significant if controlled for the WBC [115].

In addition to ATRA, arsenic trioxide (As_2O_3) and tetra-arsenic tetra-sulphide (As_4S_4) are efficacious [127], these therapies also having been pioneered in China from 1992 onwards [128]. Among chemotherapeutic agents, anthracyclines (either daunorubicin or idarubicin) are of considerable importance, whereas cytarabine may be of little or no importance [122,129]. 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 [119]. CD33-targeted monoclonal antibodies (gemtuzumab ozogamicin, 'Mylotarg') may also contribute to successful outcome [130]. The standard treatment is now ATRA plus arsenic trioxide with an anthracycline added only for high-risk cases.

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 [128]. The two drugs are synergistic. Tetra-arsenic tetra-sulphide differs in that it leads to apoptosis rather than differentiation of promyelocytes [127].

Immunophenotype

APL has a characteristic immunophenotype [54] (Fig. 3.16). This can be useful in distinguishing it from other subtypes of AML, for example 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 [131,132] whereas in another large study no difference was detected [133]. Flow cytometry shows, on light scatter measurements, a compact cluster with relatively high forward and side scatter [134]. However, high side scatter, attributable to the granularity of the cells, is not invariably increased and is less likely in the variant form [135]. There may be significant autofluorescence [136] 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 [131]. CD33 expression is homogeneous [54], whereas CD13 expression tends to be heterogeneous [54,133,137]. CD33 is characteristically more strongly expressed than in normal neutrophils [136]. HLA-DR and CD34, which are positive on early granulocytic lineage cells but negative on normal promyelocytes, are usually negative but expression is variable [136], and approaching a fifth of cases may be positive for CD34, particularly in the hypogranular variant [126,131,132,138–140]; 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 [141]. CD34 expression has been found to be an independent poor prognostic factor, as well as correlating with variant cytology and a higher WBC [140]. CD105, CD109 and CD133, other markers of immaturity, are negative [135,142]. TdT is positive in a minority of patients [133]. Negativity for CD11a and CD18 is higher than in other types of AML (81% cf. 12%) [143]. CD38 may be expressed but expression is low [135]. CD45 expression is usually weak, with stronger expression being more likely in the variant form [135]. Positive reactions for CD11b, CD11c and CD14 are seen in only a minority of cases; these reactions, more typical of monocytes, do not correlate with anomalous expression of non-specific esterase [144]. CD64, which is also characteristic of the monocytic lineage, is usually

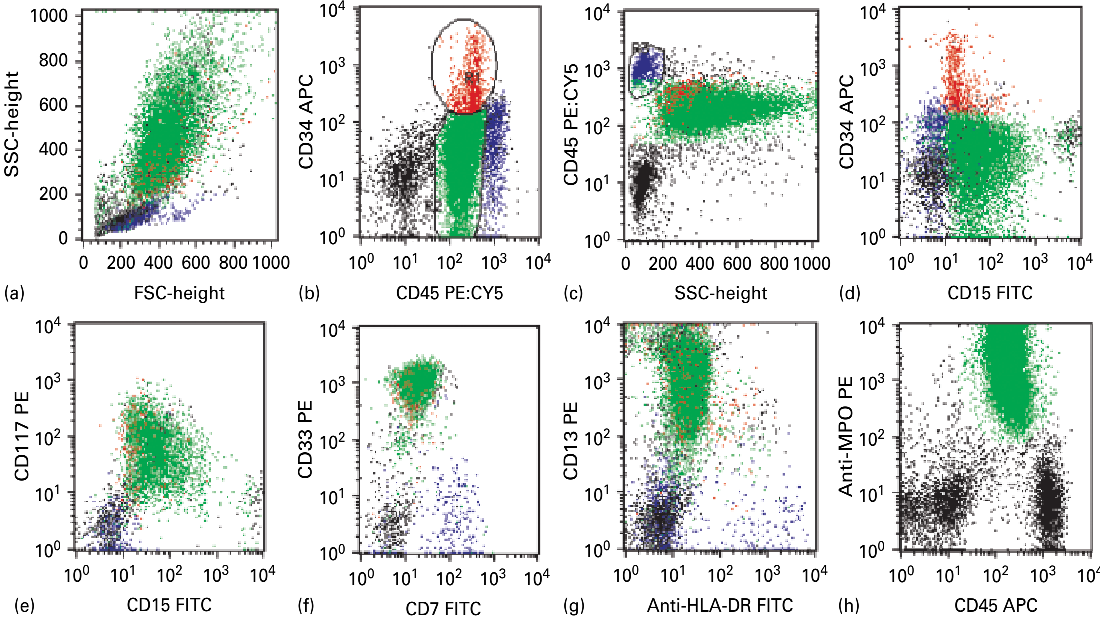


Fig. 3.16 Four-colour flow cytometry immunophenotyping from a patient with AML associated with t(15;17) (compare with Fig. 2.3). Plots a, b and c show standard analysis regions. The blast cells in this subtype of AML are usually CD34− CD45+. Their key phenotypic features are shown in plots d to h. In 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). (With thanks to Dr Steve Richards.)

positive whereas CD65 has been reported to be more often negative [56] and to be weaker in the variant form [135]. A CD65 antibody recognizing CD65s gives stronger reactions than CD65 antibodies that recognize the asialo form on the antigen [135]. Variation between antibodies is the likely explanation of the inconsistent expression of CD65 sometimes reported [137]. CD117 has been reported to be more often negative [56] but others have found CD117 to be sometimes [131,132] or often [133] positive or consistently positive but with variable strength of expression [135,137]. CD15, which is expressed by normal promyelocytes, has been reported as negative [131,137] or often positive [132]; 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 [135]. CD11a, which is often expressed in other categories of AML (but not in t(8;21)-associated AML), is not expressed [135,145]. 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 [135]. HLA-DR is, however, more often positive in the variant form [126]. CD56 is usually negative [138] and when expressed (in about 20% of patients) has been correlated with extramedullary relapse [146] and with worse outcome [135,147]. CD2, CD4 and CD19 have been reported to be positive in a significant minority of cases [60,133,139] with expression of these antigens being reported to be more likely in the variant form [126,131,138]; however, others have reported that CD19 is invariably negative [135] as is CD79a [135]. In one study, CD2 expression was associated with variant morphology and with a better response rate and

event-free survival [148] whereas, in two further studies, expression of CD2 was associated with variant morphology and a higher WBC [126,149] and with either a shorter duration of complete remission [126] or worse survival [149]. CD2 expression has been associated with *FLT3*-ITD [7]. CD24, which is positive in normal cells of neutrophil lineage from the promyelocyte stage onwards, is negative [135]. CD16 is negative. CD7 is usually negative [150]. 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 [151]. Cases of AML that are HLA-DR negative and CD2 positive are likely to be acute promyelocytic leukaemia [57]. 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 [133].

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 side scatter and weak CD45 expression. CD33 and CD117 are more consistently positive than CD13. Reactions for CD34, HLA-DR, CD11a, CD11b, CD11c and CD18 are most often weak or negative. CD2 may be positive but CD7 is negative. B-cell antigens are negative.

Imaging flow cytometry provides a potential method for detection of the microparticulate distribution of PML protein [152].

Cytogenetic and molecular genetic features

The frequency with which the specific t(15;17)(q24.1;q21.2) translocation (Fig. 3.17) is detected is method dependent since direct examination without culture may result in only non-clonal erythroid cells entering mitosis [153]. MAC techniques show t(15;17) in the granulocytic lineage but not in erythroid or megakaryocyte lineages [74]. 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 a *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 in one series, and in 9% and 36% in two others), abnormal 7q (present in up to 5% of patients), and del(9q), 8q+ and +21 (each present in 1–2% of patients) [154–156]. An isochromosome of the long arm of the derivative 17, ider(17q), which results in two copies of the *PML-RARA* fusion gene, was observed in fewer than 1% of patients [154] and in 3.6% of patients [156]; in one study, the seven

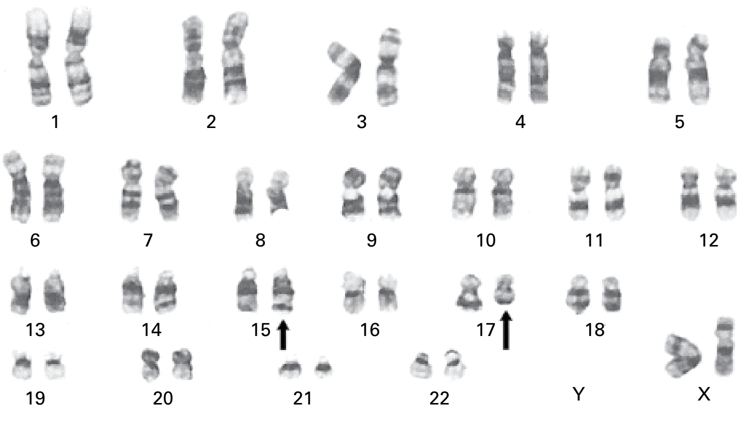


Fig. 3.17 Karyogram showing the translocation between chromosomes 15 and 17 in a female patient with FAB M3 AML and the karyotype 46,XX,t(15;17)(q24.1;q21.2). (With thanks to Professor Lorna Secker-Walker.)

cases with ider(17)(q10)t(15;17)(q22;q12) were all hypergranular rather than variant cases [138]. An adverse prognostic significance of secondary chromosomal anomalies was observed in one series of patients [157] but not in five others [49,154,155,158,159]. In a further study a single additional chromosomal abnormality was not prognostically adverse while the presence of two or more additional abnormalities was associated with an inferior overall survival [156]. Cases that lack Auer rods appear to be more likely to have additional chromosomal abnormalities [160], and in one study the prevalence of the variant form was significantly less in patients with additional chromosomal abnormalities [159]. Classical APL 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* [161]. 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 APL. In one study only a single instance was observed among 530 cases [162]; on morphological review, it was reclassified from M5 to the microgranular variant of APL.

The molecular mechanism of leukaemogenesis is fusion of part of the *PML* (promyelocytic leukaemia) gene at 15q24.1 with part of the *RARA* (retinoic acid receptor α) gene from 17q21.2 [163] 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 [164,165] (Fig. 3.18); the necessary antibody is commercially available. However, it should be noted that the distribution of PML

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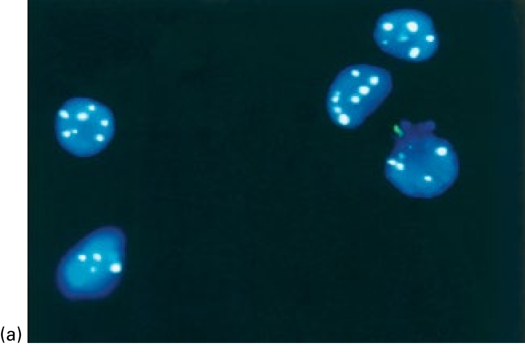


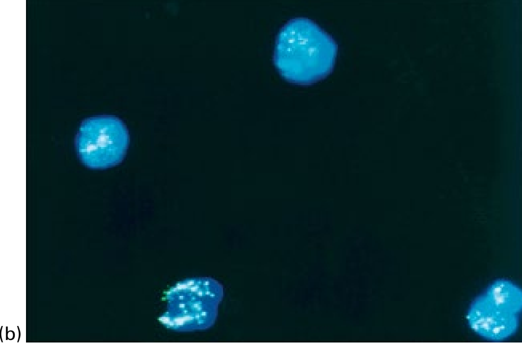
(b)


Fig. 3.18 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. (With thanks to Dr Sheila O'Connor, Leeds, and with permission of the *British Journal of Haematology* [165].)

protein is also abnormal in *NPM1*-mutated AML [166]. The fusion gene *RARA-PML* on chromosome 17 is expressed in about 70% of cases and may contribute to oncogenesis [161,167]. 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 produced the cytological features of APL but without the characteristic immunostaining pattern with anti-PML antibodies or ATRA sensitivity [168]. Very rarely no relevant abnormality is detected on standard cytogenetic analysis or FISH but *PML-RARA* is detected by RT-PCR [169]. When APL occurs as a therapy-related leukaemia the precise breakpoints in the *PML* gene are clustered and differ from those in *de novo* disease [170–172].

FLT3-ITD is seen as a second genetic event in about a third of patients [173], being particularly common in those with variant cytological features [79,115,174–176] and correlating with a higher WBC [115,174–176]. In one study *FLT3*-ITD was not an independent poor prognostic feature [175] whereas in another it correlated with worse overall survival [177], and in a third with more induction deaths but no significant difference in relapse rate or overall survival [176]. In a fourth study *FLT3*-ITD was significant for relapse but not for overall or disease-free survival [115]. With modern therapy incorporating As_2O_3 as well as ATRA, *FLT3*-ITD may not be prognostically adverse [156]. *FLT3*-ITD is also a feature of therapy-related cases [90]. *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* [77]. *NRAS* and *KRAS* mutations also occur although *NRAS* mutations are under-represented in comparison with AML in general [178]. *MYC* is overexpressed in some patients. Hypermethylation of the *CDKN2B* promoter is associated with adverse prognosis, even after allowing for the correlation with a higher WBC [179]. A polymorphism in *TNFRSF6*, encoding CD95, correlates with a worse prognosis [180].

Using microarray analysis of gene expression, AML associated with t(15;17) can be distinguished from AML with t(8;21) or inv(16)/t(16;16) [78]. In addition, cases with classical morphology can be distinguished from the microgranular variant [78].

PML-RARA rearrangement can be detected by dual-colour, single-fusion [92,181] (Fig. 3.19a) and dual-colour, dual-fusion FISH (see Fig. 2.15). Rearrangement of *RARA* can also be detected using dual-colour, break-apart FISH (Fig. 3.19b; see also Fig. 2.14). *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 [182].

Detection of MRD, by RT-PCR, after consolidation therapy is of prognostic significance [122,183]. Treatment of molecular relapse appears to give superior results to waiting for haematological relapse [122]. 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 [95]. 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 [183]; however, transplantation is now rarely used in this subtype of leukaemia.

Acute myeloid leukaemia with t(11;17)(q23.2;q21.2); *ZBTB16-RARA* or other *RARA* rearrangement

In addition to t(15;17)(q24.1;q21.2), there are other translocations associated with rearrangement of the *RARA* gene, with leukaemic cells often having some features reminiscent of APL. However, many of these cases show cytological and clinical differences from AML with *PML-RARA* fusion. These molecular variants, the most common of which is t(11;17)(q23.2;q21.2),

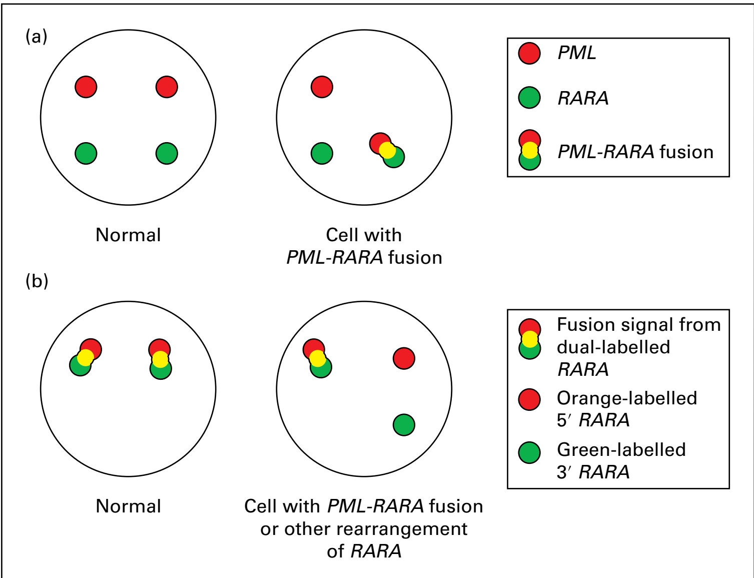


Fig. 3.19 Diagrammatic representation of two FISH strategies for the detection of *RARA* rearrangements. (a) Dual-colour, single-fusion FISH technique in t(15;17)(q24.1;q21.2). The normal cell has two red *PML* signals and two green *RARA* signals. The abnormal cell has one normal red *PML* signal, one 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)(q24.1;q21.2) or variant translocations such as t(11;17)(q23.2;q21.2). 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.

are summarized and compared with *PML-RARA*-related cases in Table 3.2. In the WHO classification, these cases should be diagnosed as acute promyelocytic leukaemia with a variant *RARA* translocation.

In addition to cases with variant *RARA* translocations, a case of AML morphologically and immunophenotypically resembling acute hypergranular promyelocytic leukaemia has been described in association with t(11;12)(p15;q13) and a *NUP98-RARG* fusion gene, *RARG* being another member of the RAR receptor family [197].

Clinical and haematological features

In AML associated with t(11;17)(q23.2;q21.2); *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. 3.20); 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 coloration characteristic of M3 AML; giant granules are sometimes present. The nucleus is usually round rather than irregular or bilobed, there is more chromatin condensation and maturing granulocytes may include Pelger–Huët forms [138]. In one patient with t(5;17) the cytological features were more similar to APL but Auer rods were lacking [138], whereas two previously reported cases were associated with classical and variant morphology, respectively. The t(11;17)(q23.2;q21.2); *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

Table 3.2 Characteristics of acute myeloid leukaemia (AML) associated with rearrangement of the *RARA* gene [138,167,184–196].

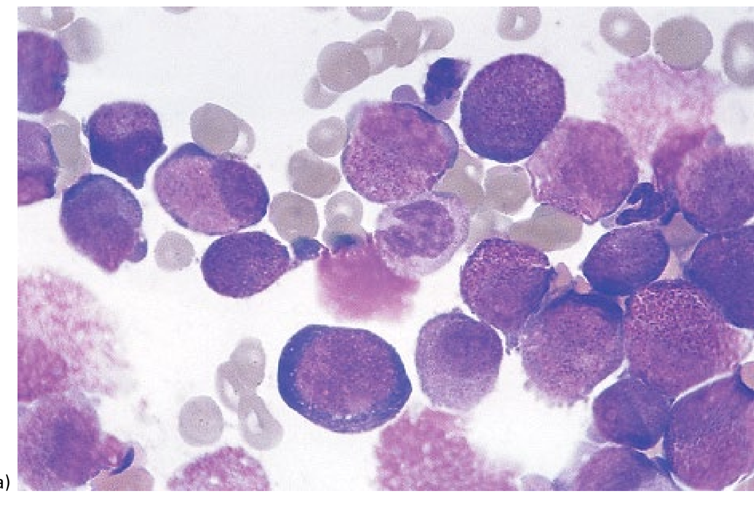
Cytogenetics	Molecular genetics	Responsiveness to ATRA	Distribution of PML protein [167,187,196]	Frequency
t(15;17)(q24.3;q21.2)	*PML-RARA*	Yes	Abnormal (i.e. microparticulate)	About 91% of FAB M3 and M3-like AML
Simple or complex variant translocations, ider(17), ins(15;17) or insertional events	*PML-RARA*	Yes	Microparticulate	About 7% of FAB M3 and M3-like AML
Cryptic ins(17;15)*	*RARA-PML*	No	Normal (i.e. discrete nuclear bodies)	
t(11;17)(q23.2;q21.2) (or normal with *RARA* insertion into 11q23)	*ZBTB16-RARA*	No†	Inconsistent reports of normal (discrete nuclear bodies) or microparticulate	About 0.8% of FAB M3 and M3-like AML
t(11;17)(q13.4;q21.2)	*NUMA1-RARA*	Yes	Mainly cytoplasmic [196]	At least three patients
t(5;17)(q35.1;q21.2)	*NPM1-RARA*	Yes	Inconsistent results reported [196]	At least 10 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* is at 17q21.2)	*STAT5B-RARA*	No	Microparticulate [196]	At least nine patients
+22 and cryptic del(17)(q21); normal; t(17;17)(q24;q12)	*PRKAR1A-RARA*	Yes	Normal	Rare
t(4;17)(q12;q21)	*FIP1L1-RARA*	Yes	Not known	Two patients [190,196]
t(X;17)(p11.2;q12)	*BCOR-RARA*	Yes‡	Diffuse ± cytoplasmic	Two patients [193,196]
t(2;17)(q32;q21)	*NABP1 (OBFC2A)-RARA*	Probably	Not known	One patient [194]
t(3;17)(q26;q21)	*TBL1XR1-RARA* also known as *TBLR1-RARA*	Uncertain	Diffuse in nucleus and cytoplasm	Three patients [195]
Cryptic t(7;17)(q11;q21)	*GTF2I-RARA*	No	Microparticulate	[196]
t(1;17)(q42;q21)	*IRF2BP2-RARA*	Yes	Not known	[196]

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

* Morphologically not hypergranular promyelocytic leukaemia.

† Although lack of responsiveness is not absolute [192]; also unresponsive to arsenic trioxide therapy [188].

‡ Resistant to arsenic trioxide


(a)

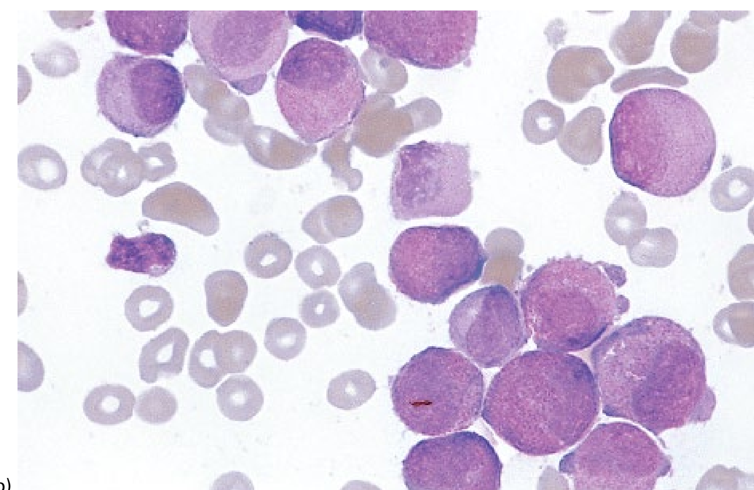

(b)

Fig. 3.20 BM film from a patient with FAB M2/M3 AML with t(11;17)(q23.2;q21.2) 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. (With thanks to Dr Dominic J. Culligan, Aberdeen.)

suggestive of M3 variant AML [187]. A second patient was categorized as M3 AML [189]. A single patient with *PRKAR1A-RARA* fusion was morphologically M3 AML but without Auer rods [191]. AML associated with t(X;17)(p11.2;q12) had unique cytological features with M2-M3 morphology, nuclei of regular shape, and rectangular and round PAS-positive cytoplasmic inclusions [193]. The t(3;17)(q26;q21) translocation was associated with acute hypergranular promyelocytic leukaemia [195].

The prognosis of M3-like AML associated with t(11;17)(q23.2;q21.2) is poor [48].

Immunophenotype

The immunophenotype of AML with t(11;17)(q23.2;q21.2); *ZNF145-RARA* AML is similar to that of AML with *PML-RARA* [138]. There is usually expression of CD13 and CD33 and lack of expression of HLA-DR and CD34 [133,138]; CD56 is much more often expressed [138].

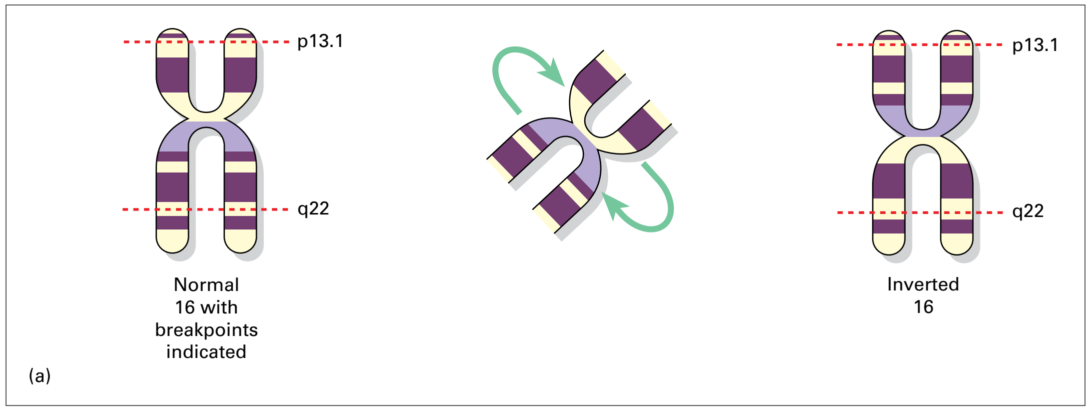
Cytogenetic and molecular genetic features

All these molecular variants involve chromosome 17 and the *RARA* gene but the partner chromosome and gene differ. In t(11;17)(q23.2;q21.2) part of the *ZBTB16* gene – previously known as

ZNF145 (zinc finger 145) and as *PLZF* (promyelocytic leukaemia zinc finger) – at 11q23.2 fuses with the *RARA* gene [184,186] 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 [186]. 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)(q23.2;q21.2) [138]. 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. 3.19b).

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 [198] although Arthur and Bloomfield had, shortly before, described AML with abnormal eosinophils in association with what they interpreted as del(16)(q22) [199]. Inversion of chromosome 16 (Fig. 3.21a) and the less common reciprocal translocation between the chromosome 16 pair (Fig. 3.21b) are associated with AML with identical features [200,201] although t(16;16) was associated with a worse prognosis in one study [202]. Such cases



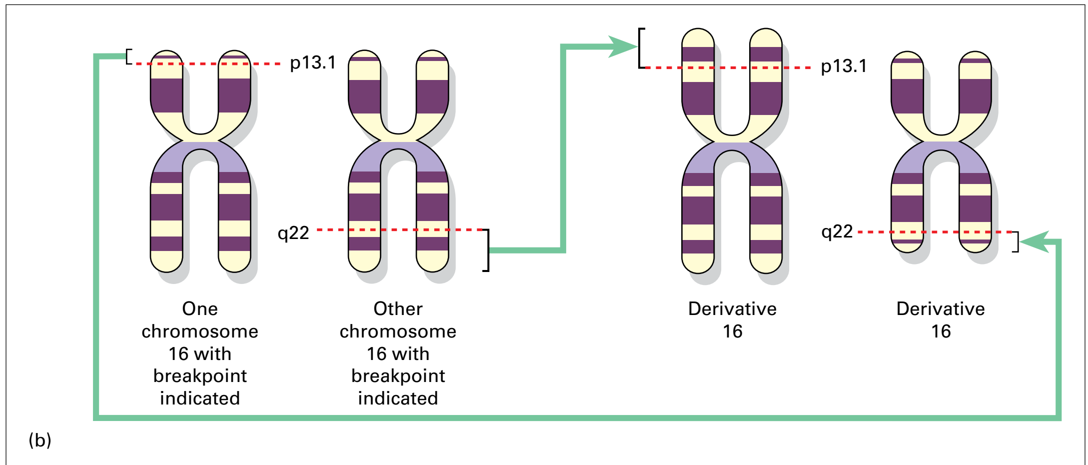


Fig. 3.21 (a) A diagrammatic representation of inv(16)(p13.1q22); this is an example of a pericentric inversion (modified from reference [2]). (b) A diagrammatic representation of t(16;16)(p13.1;q22).

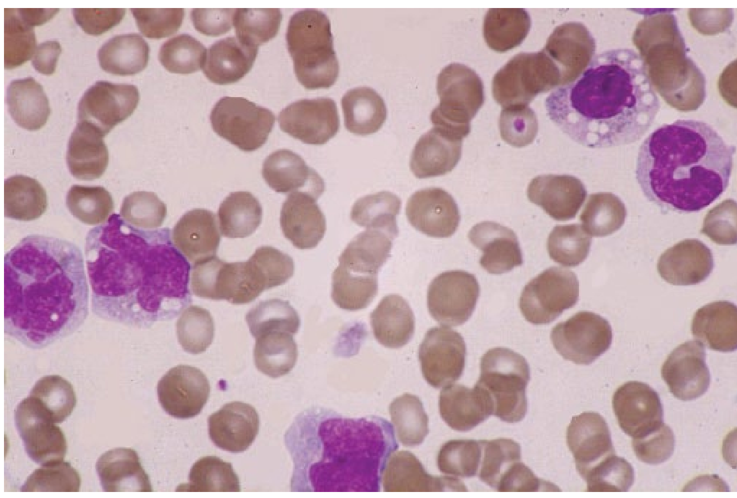
comprise 3–8% of AML with inv(16) being about five times as common as t(16;16); in a series of 1897 unselected adult patients prevalence was 4.6% [17]. The frequency is slightly higher in children than in adults [18], and in elderly adults the prevalence falls to 1% of cases of AML [19]. The prevalence is significantly higher among t-AML than among *de novo* AML [17], with therapy-related cases representing *c.* 14% of all cases [202]. Therapy-related cases are not assigned to this category but are categorized with other therapy-related myeloid neoplasms; they are recognized following exposure to topoisomerase II-interactive drugs and other agents [22,23]. Prognosis is worse in therapy-related cases [203] but nevertheless, 5-year survival approaches 50% and is thus considerably better than in most other types of t-AML [109].

This subtype of leukaemia may have an intrauterine origin with a latency of about 10 years [107]. A unique case has been reported in which lineage switch to B-lineage acute lymphoblastic leukaemia (ALL) occurred at relapse [204]. This inversion retains a good prognostic significance in patients in first relapse and is incorporated into the European Prognostic Index [47].

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 [153,198,205] (Figs 3.22–3.26; 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 [206]. Bone marrow eosinophils are less than 5% in more than a quarter of patients [207]. Nevertheless, morphologically abnormal cells of eosinophil lineage are probably always present, although they may constitute as few as 0.2% of bone marrow cells [48,207]. 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, including of the bowel or bladder, occurring in advance of overt leukaemia or as the first sign of relapse. There is a significant association with a tumour lysis syndrome [208]; this may be related to disease burden since six reported patients had a median WBC of 147×10^9/l. Patients are relatively young (median age 34 years) [201].

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



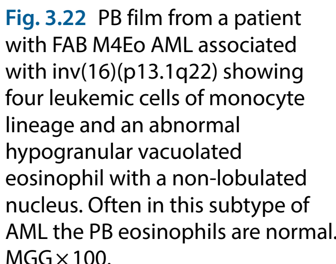


Fig. 3.22 PB film from a patient with FAB M4Eo AML associated with inv(16)(p13.1q22) showing four leukemic cells of monocyte lineage and an abnormal hypogranular vacuolated eosinophil with a non-lobulated nucleus. Often in this subtype of AML the PB eosinophils are normal. MGG × 100.

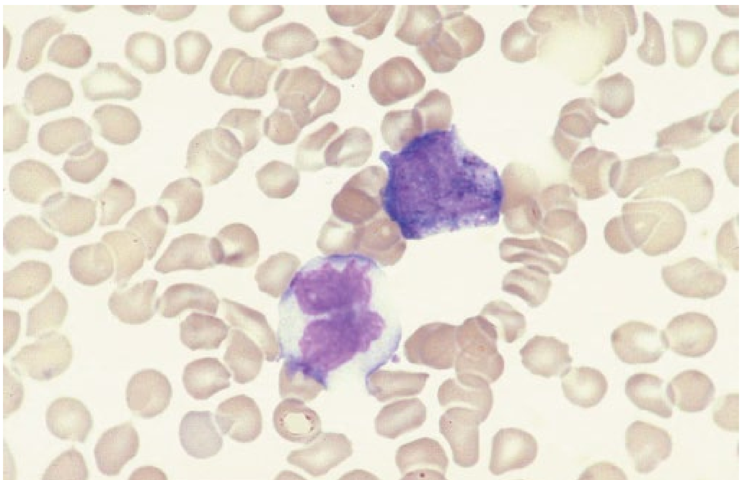
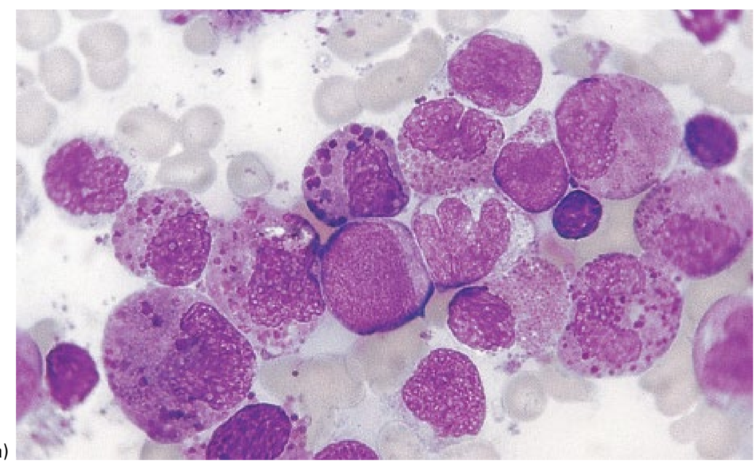


Fig. 3.23 PB film from a patient with FAB M4Eo AML associated with t(16;16)(p13.1;q22) showing an abnormal cell of monocyte lineage and an eosinophil precursor that has a mixture of granules with eosinophilic and basophilic staining characteristics. MGG × 100.

(a)



(b)

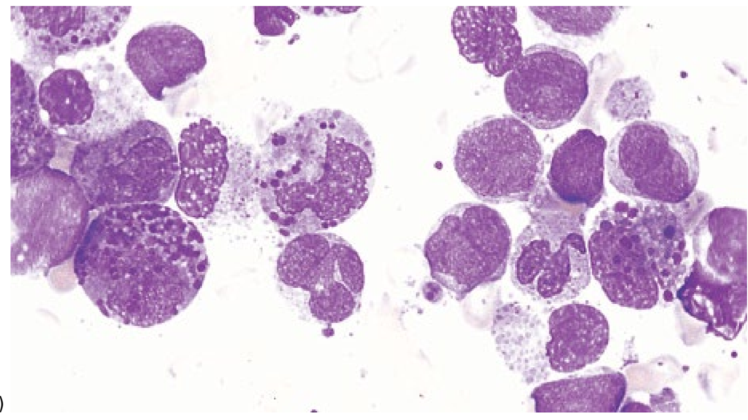


Fig. 3.24 BM film from a patient with AML FAB M4Eo/inv(16)(p13.1q22). (a, b) MGG stain showing abnormal blasts, monocytes, mature eosinophils and eosinophil myelocytes with abnormally basophilic granules. MGG × 100.

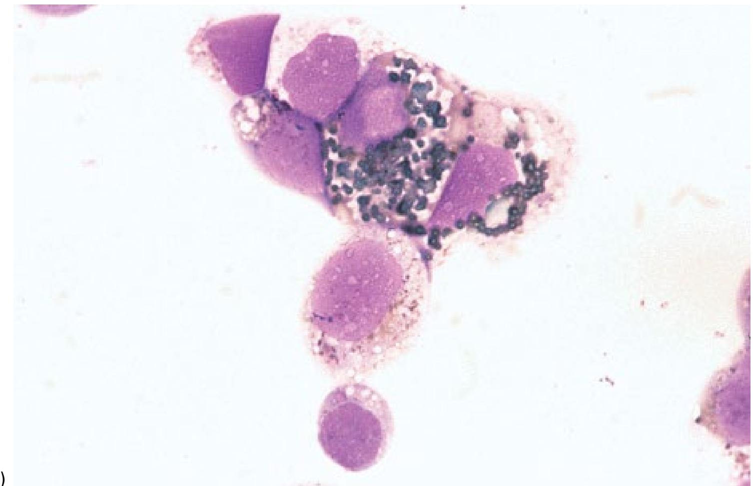

(c)

Fig. 3.24 (*Continued*) (c) SBB stain showing large abnormal granules in the eosinophil lineage and occasional small granules in monocytes. SBB × 100.

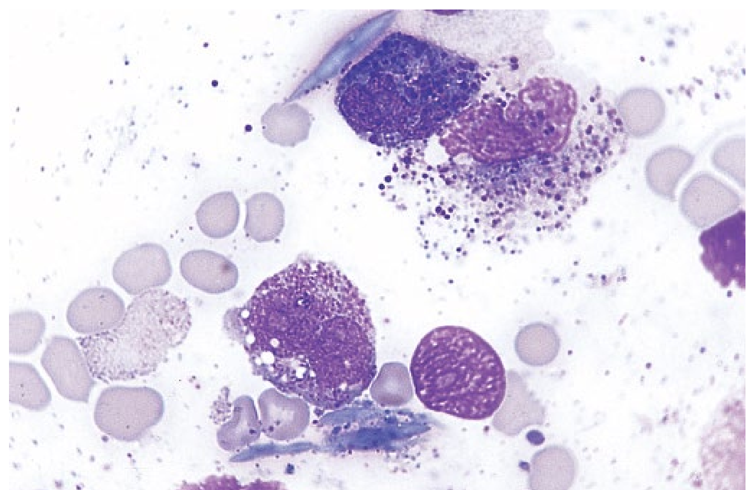


Fig. 3.25 BM film from a patient with AML M4Eo/inv(16)(p13.1q22) showing Charcot–Leyden crystals and abnormal cells of eosinophil lineage. MGG × 100. (With thanks to Dr Ralph Cobcroft and Dr Devinder Gill, Brisbane.)

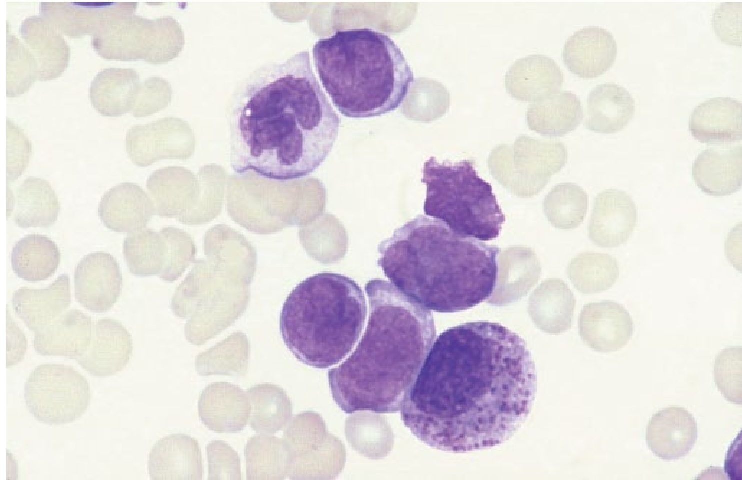


Fig. 3.26 BM film from a patient with FAB M4Eo AML and t(16;16)(p13.1;q22) showing one abnormal monocyte, four blast cells and an eosinophil precursor with granules with mixed eosinophilic/basophilic staining characteristics. MGG × 100.

blasts cell are sometimes less than 20%; such cases are nevertheless categorized as AML [7]. Auer rods are usually present although only in a minority of cells; they are sometimes present in mature neutrophils. Rarely bundles of Auer rods, more characteristic of acute promyelocytic leukaemia, are seen [209]. Neutrophils may be cytologically abnormal [207]. Bone marrow eosinophils and to a greater extent eosinophil myelocytes show prominent proeosinophilic granules, which are basophilic in their staining characteristics and larger than normal (see Fig. 3.22). 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 [12] and sometimes in reactive eosinophilia [153]. Positivity for CAE may, however, indicate that eosinophils are part of a leukaemic process [153]. 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) [210] (see Fig. 1.30). Despite the monocytic differentiation, nonspecific esterase reactions are often weak [207]. 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 [211].

The prognosis of this category of AML is relatively good, with reported complete remission rates of 85–93% [46,201] and 5-year survivals of 50–60% [19,44–46,201]. A series of 266 patients entered into MRC trials had a median 10-year survival of 55% [49]. Prognosis is significantly better in those with a presenting WBC of 20×10^9/l or less [51] and is worse in those with a WBC above 120×10^9/l or a platelet count below 30×10^9/l [201]. The prognosis in children appears to be equally good [48]. 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 [201]. Intensive post-remission intensification with high-dose cytarabine was thought to contribute to improved survival in one trial [46] but not in another [201].

Immunophenotype

Flow cytometric immunophenotyping shows both granulocytic and monocytic differentiation [54] (Fig. 3.27). The plot of side scatter against forward scatter may show a characteristic forked pattern [136]. 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 [212]. 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 side scatter and expression of CD13, CD33, CD65, MPO and strong CD15 [136]. CD2 is expressed in about 40% of patients and correlates with CD11b and CD14 expression [59,135,145]. CD19 may be expressed. CD34 is expressed in the great majority of cases [213,214]. TdT is more often positive than in AML in general [215]. CD7 is not usually expressed [214]. Strong expression of CD34 and CD13 and weak expression of CD33 has been reported to have a reasonably high sensitivity and specificity [215]. This category of leukaemia has also been identified by flow cytometry, using an antibody directed at the CBFB-MYH11 fusion protein [216].

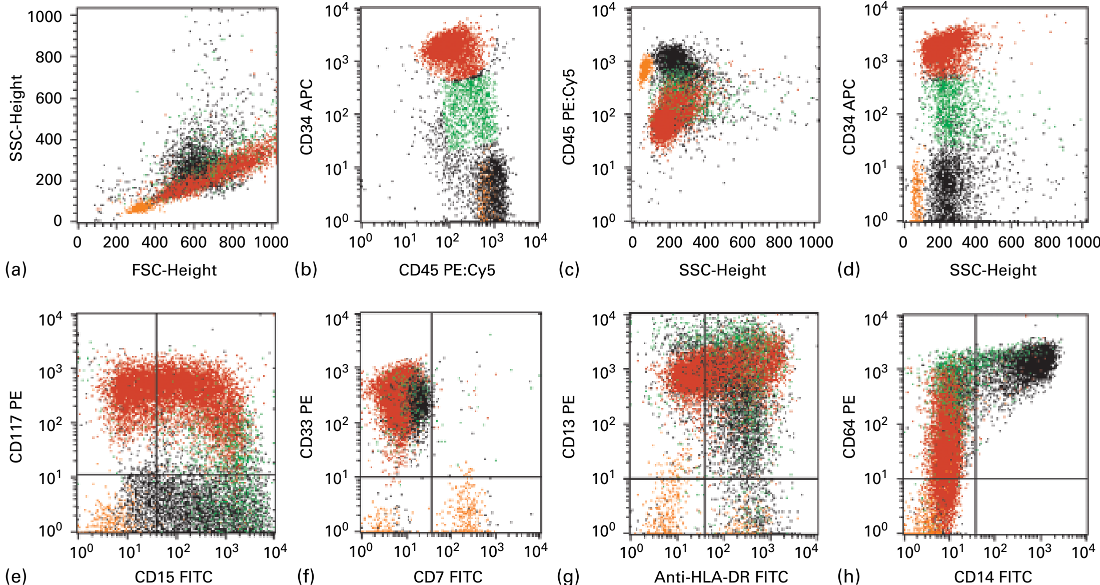


Fig. 3.27 Four-colour flow cytometry immunophenotyping in a patient with AML associated with inv(16) (compare with Fig. 2.3). Plots a–d show distinct CD34++, CD34+ and CD34– blast cell populations. Plots e and h show a spectrum of differentiation, with the most immature blast cell component (plot e) 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). (With thanks to Dr Steve Richards.)

Cytogenetic and molecular genetic features

M4Eo AML is associated both with inv(16)(p13.1q22) (Fig. 3.28) and, less often, t(16;16)(p13.1;q22) (Fig. 3.29). 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. In a large series of patients ($n = 176$), trisomy 22 was found in 18% and trisomy 8 in 16% [203]. The presence of secondary cytogenetic abnormalities, even complex ones, did not appear to worsen prognosis in one study [45] but, in another, trisomy 21, present in about 4% of patients, was associated with shortened survival [202]; in a third, trisomy 22 was a favourable feature [217]; 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 associated with a worse prognosis [46]. In a study of outcome in 266 patients entered into MRC trials, a lower WBC and a significantly longer survival were found in patients with additional abnormalities, including specifically those with trisomy 22 [49]. In a second large series of 176 patients entered into a German-Austrian study, trisomy 22 was confirmed, on multivariate analysis, as a favourable factor for overall and relapse-free survival, while trisomy 8 was of adverse prognostic significance for overall survival [203]. 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 [218]. M4Eo AML has also been described in association with a deletion of chromosome 16, del(16)(q22) [199].

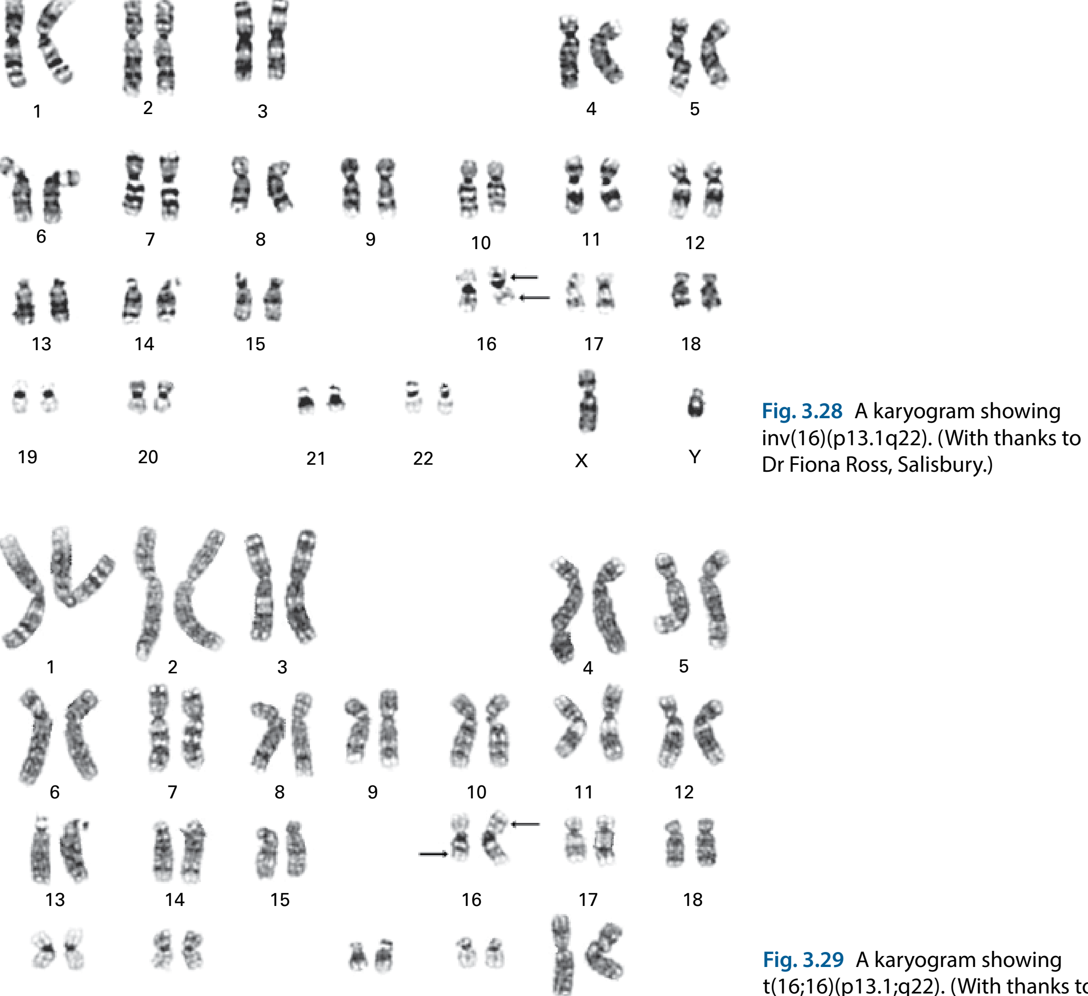


Fig. 3.28 A karyogram showing inv(16)(p13.1q22). (With thanks to Dr Fiona Ross, Salisbury.)

Fig. 3.29 A karyogram showing t(16;16)(p13.1;q22). (With thanks to Dr Fiona Ross.)

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 [153,219] and with a high incidence of preceding MDS [219]. 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 [153]. 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). In patients presenting with myeloid sarcoma, cytogenetic analysis of bone marrow cells may show inv(16) in a proportion of metaphases, even in the absence of cytological evidence of bone marrow disease [220]. 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 16p13.1 to form a fusion gene, *CBFB-MYH11*, which encodes a protein that interferes with normal control of transcription [221]. 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 [170]. A minority of patients have submicroscopic deletion of part of chromosome 16, telomeric to the *CBFB* gene [222]; this may be associated with a worse prognosis. A *KIT* mutation is found as a secondary abnormality in 30–40% of patients with inv(16), some of whom have aberrant bone marrow mast cells [7,86]; such mutations correlated with a worse prognosis in several series of patients [84,203] but not in all [83]. *KIT* and *RAS* mutations are both more common than in AML in general [88–90]. *NRAS* mutation, present in 37% of patients, did not influence prognosis [90]. *FLT3*-ITD is uncommon [79,84,178]. In one large study *FLT3*-ITD was found in 7% of patients and *FLT3*-TKD mutation in 24% [81]. In a further very large study (176 patients) the frequency of mutations was: *NRAS* 45%, *KIT* 37%, *FLT3* 17% (14% *FLT3*-TKD, 5% *FLT3*-ITD, with both in 2%) and *KRAS* 13% [203]; on multivariate analysis, adverse prognostic significance was found for *KIT* mutation for relapse-free survival and for *FLT3* mutation (mainly *FLT3*-TKD) for overall survival [203]. In another large study a *FLT3*-TKD mutation was found in 13% of 420 patients and did not have a statistically significant influence on survival [223].

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) [78].

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 [224], by dual-colour, break-apart FISH using a 5′ *CBFB*–3′ *CBFB* probe (Figs 3.30 and 3.31), 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

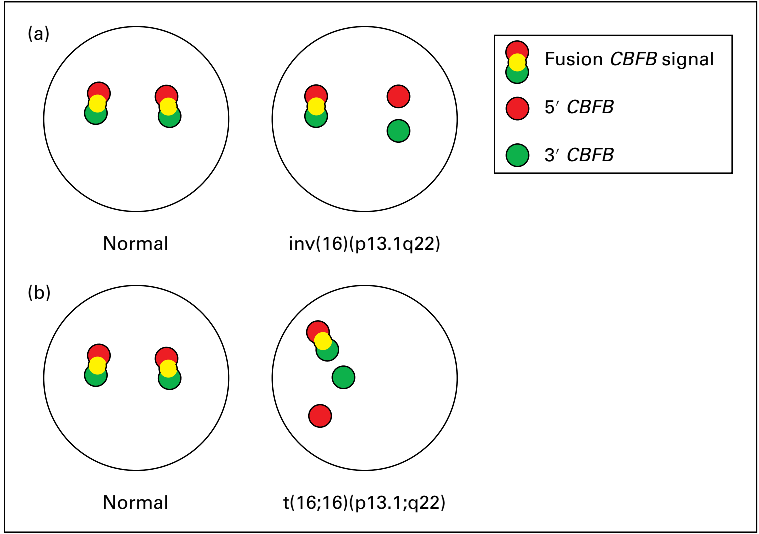


Fig. 3.30 Diagrammatic representation of a dual-colour, break-apart FISH technique for detecting the disruption of *CBFB* in inv(16)(p13.1q22) and t(16;16)(p13.1;q22), using a red-labelled probe for 5′ *CBFB* and a green-labelled probe for 3′ *CBFB*: (a) a normal cell has two yellow fusion signals whereas 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.

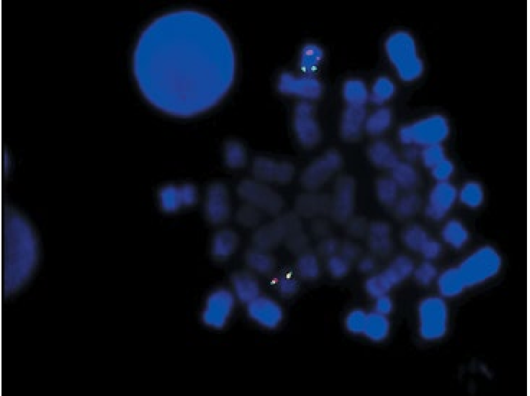


Fig. 3.31 FISH of a metaphase from a patient with inv(16)(p13.1q22) using a dual-colour, break-apart probe; the 5′ *CBFB* probe (centromeric to the breakpoint) is labelled red and the 3′ *CBFB* probe (telomeric to the breakpoint) is labelled green. Normal cells will thus have two fused red–green (yellow) 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. (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

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 [225]. A significant proportion of patients (varying from less than 10% to 50% in different series of patients [226]) 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 [16], and another group also found no discrepancy [227]. When molecular techniques are employed the prevalence of this subtype among cases of AML may be as high as 10% [205]. 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 [228,229]. Using RQ-PCR, the quantity of fusion gene transcript present at presentation and after consolidation chemotherapy is predictive for event-free survival [95]. A 1 log or greater increase in transcript from the remission level is predictive of relapse [96]. Molecular relapse may precede haematological relapse by many months [230].

Acute myeloid leukaemia with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*

There is a strong association between acute monoblastic/monocytic leukaemia and deletions or translocations with a breakpoint within the *KMT2A* gene (a homologue of the *Drosophila trithorax* gene previously known as *MLL*, *HRX*, *ALL-1* and *Htrx-1*) at 11q23.3 [231]. Overall such cases comprise about 3–4% of adult AML cases [15] but about 18% of childhood cases [18]; in a series of 1897 unselected adult patients, prevalence was 2.8%, being significantly higher among t-AML cases [17]. In another series of patients about 8% of those with t(9;11) had AML secondary to topoisomerase II-interactive drugs [232]. Therapy-related cases are not assigned to this category but rather are classified as t-AML. The prognosis of therapy-related cases is worse than that of *de novo* cases, with less than 20% 5-year survival [17,233]. Of the myeloid leukaemias in which there is a chromosomal rearrangement with an 11q23.3 breakpoint, that associated with t(9;11)(p21.3;q23.3) and *KMT2A-MLLT3* is the most common.

Clinical and haematological features

The prevalence of AML with t(9;11)(p21.3;q23.3) 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) [18]. 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 [234]. There may also be soft tissue infiltration (gums and skin) or DIC [7]. Most cases of AML with t(9;11) are FAB M5a AML (Fig. 3.32) but some are M5b or M4 (Fig. 3.33), and occasional cases are M1, M2

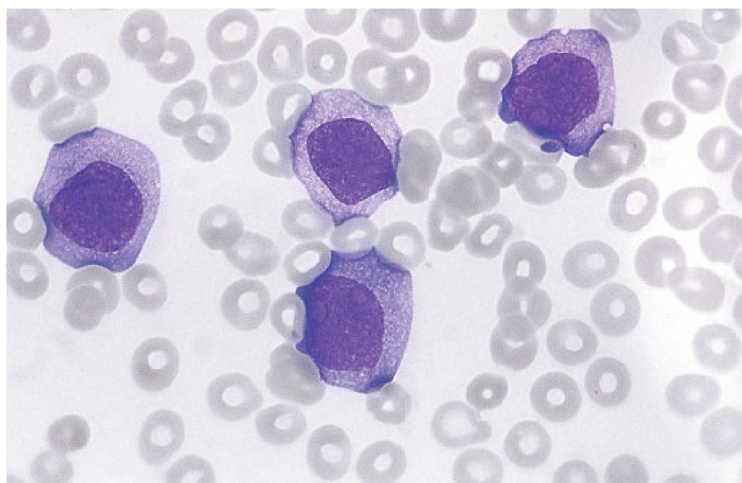


Fig. 3.32 PB film from a patient with FAB M5a AML associated with t(9;11)(p21.3;q23.3). MGG × 100.

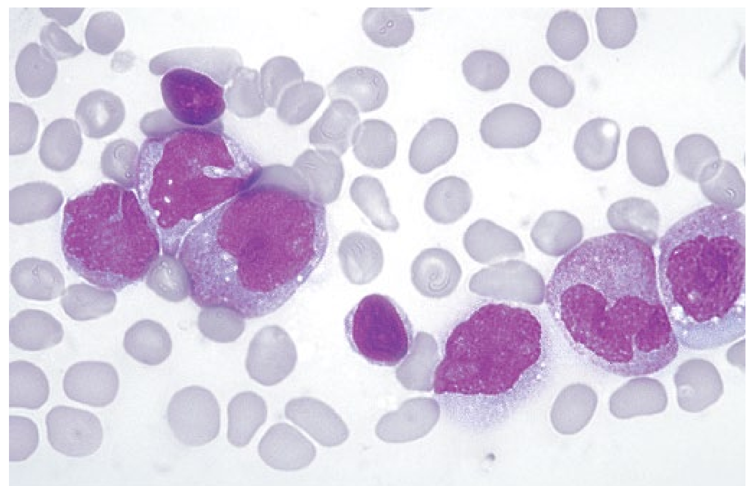


Fig. 3.33 BM film from a patient with FAB M4 AML associated with t(9;11)(p21.3;q23.3). MGG × 100. (With thanks to colleagues in the European 11q23 Workshop [232].)

or M7 [232]. Cytological and cytochemical features are those expected for these FAB categories. Auer rods are quite uncommon. Two patients with either t(9;11) or *KMT2A-MLLT3* have been considered to have acute leukaemia of dendritic cell origin [235]. 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 [236,237]. 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 [17,45]. Remission rate is 80–85% but with a 5-year survival of around 20–40% [45,46,238]. 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 [17]. 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 11q23.3 breakpoint, had a

worse outcome [49]. A French study confirmed the poor prognosis of t(6;11) and found a better prognosis for children with t(9;11) in comparison with adults [238]. 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 [88]. 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 [88]. Survival in patients with *KMT2A* translocations may be better with high-dose than with standard-dose daunorubicin [239].

Sustained spontaneous remission associated with sepsis has been reported [240].

Immunophenotype

The immunophenotype is characteristic of M5 AML. CD4, CD15, CD33, CD36, CD64, CD65, HLA-DR and lysozyme are usually expressed. CD13 was found to be usually negative in three series of patients [56,227] but positive in two-thirds of patients in another [232]. Similarly, CD14 was usually negative in three series of patients [56,227] but was positive in half the cases in a fourth series [232], being more likely to be negative when monoblasts were predominant. CD11b and 11c are often expressed [232], and there may also be expression of lysozyme [7]. CD34 and CD7 were positive in 40–50% of cases in one study [232], whereas three further series found CD34 expression in less than 30% of patients [227]. CD117 and CD56 are sometimes positive [7]. An NG2 homologue, a chondroitin sulphate proteoglycan encoded by *CSPG4*, is expressed. This immunophenotypic marker (detected with the 7.1 monoclonal antibody) was initially reported to be expressed in AML associated with t(9;11) or other *KMT2A* rearrangement but not in AML without *KMT2A* rearrangement [241]. However, subsequently expression, together with CD56 expression, was demonstrated in cases with monocytic differentiation but without *KMT2A* rearrangement [242], so that it does not appear very diagnostically useful.

Cytogenetic and molecular genetic features

t(9;11)(p21.3;q23.3) is difficult to detect by conventional cytogenetic analysis since the alteration in the banding pattern is subtle (Figs 3.34 and 3.35). Detection is facilitated by dual-colour, break-apart FISH with a *KMT2A* 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 [234,243,244]. *KMT2A* rearrangements can be detected by Southern blotting and *KMT2A-MLLT3* fusion by RT-PCR. t(9;11) can also be detected by

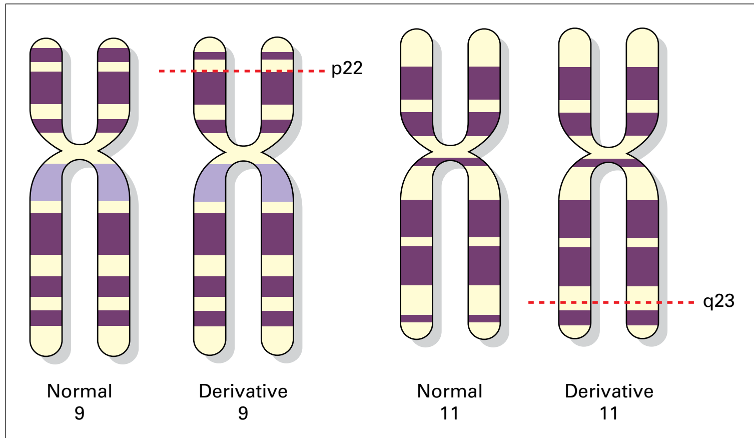


Fig. 3.34 Diagrammatic representation of t(9;11)(p21.3;q23.3) (modified from reference [2]).

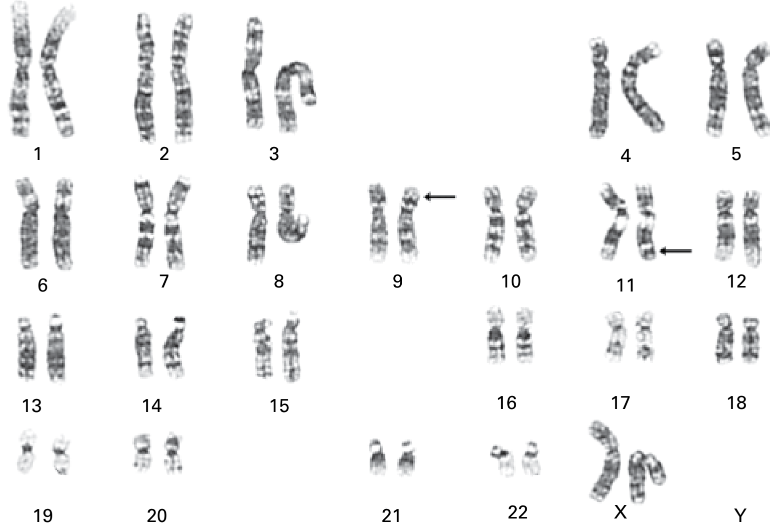


Fig. 3.35 A karyogram showing t(9;11)(p21.3;q23.3). (With thanks to Dr Fiona Ross.)

two-colour FISH using probes for *KMT2A* and *MLLT3*. *FLT3* mutations are less common than in AML in general [79]. *MECOM* (previously known as *EVI1*) is often overexpressed and may be associated with an adverse prognosis [7].

Acute myeloid leukaemia with other *KMT2A* rearrangement

In the 2016 revision of the WHO classification, other subtypes of AML with a balanced translocation and *KMT2A* rearrangement are regarded as distinct from AML with *KMT2A-MLLT3*. Unless they are therapy-related or have myelodysplasia-related changes, they are specified according to the translocation and the fusion gene formed. Rarely presentation is with a myeloid sarcoma with the bone marrow being normal, as in a patient with t(11;19)(q23.3;p13.1) and *KMT2A-ELL* [245]. The more common of these are shown in Table 3.3 [246–250] and other less common subtypes in Table 3.4, [48,236,246, 248,249,251–268] although this table is not exhaustive. More than 120 different translocations and 79 partner genes have been described [7]. One reported case of AML with t(10;11) was considered to be of dendritic cell origin [235]. These subtypes of AML with an 11q23.3 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 (such cases being classified as t-AML). Congenital cases have been reported in association with t(4;11)(q21.3;q23.3) (Fig. 3.36), t(10;11)(p13;q23.3) and t(11;19)(q23.3;p13.1) (Fig. 3.37) [269]. Features that differ between categories include the relative proportions of AML, ALL and mixed phenotype acute leukaemia (MPAL), the relative proportions of *de novo* and therapy-related cases, the proportion of patients presenting with MDS, and the relative frequency of different FAB subtypes of AML [246–268]. Prognosis also differs but is generally poor [45,270]. The prognosis of AML associated with t(6;11)(q27;q23.3) is poor [49,271,272], whilst the prognosis in cases with t(10;11)(p12;q23.3) is variously reported as poor or intermediate [37,273]. In a large series of paediatric patients, t(1;11)(q21;q23.3) was associated with a good prognosis whereas a poor prognosis was seen with t(10;11)(p12;q23.3) and t(10;11)(p11.2;q23.3) [272].

Table 3.3 Characteristics of acute leukaemia and related conditions associated with translocations and deletions involving 11q23.3 studied at the European 11q23 Workshop (percentages are shown in parentheses) [234,246–250].

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)(21.3;q23.3)	183	63 (34)	36 (20)	173 (95)	6 (3)	M4	4 (2.2)	Nil	10 (5.5)	[246]
t(6;11)(q27;q23.3)	30	2 (7)	6 (20)	3 (10)	27 (90)	M4/M5a	Nil	Nil	Nil	[247]
t(9;11)(p21.3;q23.3)	125	21/123 (17)	46/123 (37)	9 (7)	108 (86)	M5a	3 (2)	5 (4)	10 (8)	[234]
t(10;11)(p12;q23.3)	20	6/19 (32)	10/19 (53)	4 (20)	15 (75)	M5a	1 (5)	Nil	1 (5)	[248]
t(11;19)(q23.3;p13.1)	21	3 (14)	1 (5)	Nil	19 (90)	M4	Nil	2 (10)	7 (33)	[249]
t(11;19)(q23.3;p13.3)	32	13 (41)	7 (22)	21 (66)	7 (22)	M4 or M5a	4 (12)	Nil	Nil	[249]
del(11)(q23)	57	3 (5)	10 (33)	27 (47)	16 (28)	M4, M5a, M5b	2 (4)	12 (21)	1 (2)	[250]

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 = mixed phenotype acute leukaemia, acute stem cell and acute unclassified leukaemia.

Table 3.4 Further subtypes of acute myeloid leukaemia* with an 11q23.3 breakpoint involving the *KMT2A* gene (previously known as *MLL*)[†] [48,236,246,248,249,251,252,260–264,267,268]; earlier names of partner genes are shown in parentheses.

Translocation or other rearrangement	Type of leukaemia	Molecular event	Reference
ins(11;X)(q23.3;q28q12)	FAB M3	*KMT2A-FLNA*	[251]
t(1;11)(p32;q23.3)[‡]	FAB M5	*KMT2A-EPS15* (*AF1p*)	
t(1;11)(p36;q23.3)	FAB M5 (congenital)		[252]
t(1;11)(q21;q23.3)[‡]	FAB M4 (infants)	*KMT2A-MLLT11* (*AF1q*)	
t(2;11)(p21;q23.3)		Not known	
t(3;11)(p21;q23.3)	t-AML (FAB M5)	*KMT2A-NCKIPSD* (*AF3p21*)	
t(3;11)(q25;q23.3)	t-AML (FAB M4)	*KMT2A-GMPS*	[253]
t(3;11)(q28;q23.3)	A case of FAB M5 AML (t-AML)	*LPP-KMT2A* and *KMT2A-LPP*	[254]
t(4;11)(p12;q23.3)	t-AML	*KMT2A-FRYL* (*AF4p12*)	[255]
t(4;11)(q21;q23.3)	FAB M4 (more often ALL)	*KMT2A-AFF3* (*MLLT2*)	[246]
t(4;11)(q31;q23.3)		Not known	
t(6;11)(q21;q23.3)		*KMT2A-FOXO3* (*AF6q21*)	
t(6;11)(q27;q23.3)[‡]	FAB M4 or M5	*KMT2A-AFDN* (*MLLT4, AF6*)[§]	
t(8;11)(q24;q23.3)	FAB M5	Not known	[256]
t(10;11)(p12;q23.3)[‡]	FAB M5a	*KMT2A-MLLT10* (*AF10*)	[248, 257]
t(10;11)(p11.2;q23.3)	AML	*KMT2A-ABI1* (*SSH3BP1*)	
t(10;11)(q22;q23.3)	FAB M4		[258]
ins(10;11)(p11;q23.3q13-24)		Not known	
ins(11;9)(q23.3;q34)inv(11)(q13q23.3)	A case of FAB M4 AML	*KMT2A-FNBP1* (*FBP17*)	[258]
t(11;11)(q13;q23.3)		Not known	
inv(11)(p15q23.3)		Not known	
inv(11)(q14.2q23.1)	A case of AML	*KMT2A-PICALM* (*CALM*)	[259]
t(11;12)(q23.3;p13)		Not known	
t(11;14)(q23.3;q24)	A case of FAB M5 AML	*KMT2A-GPHN* (*gephyrin*)	[260]
t(11;15)(q23.3;q12)		Not known	
t(11;15)(q23.3;q14)	AML, ALL, t-MDS	*KMT2A-KNL1* (*CASC5, AF15q14*)	[261]
t(11;16)(q23.3;p13)		*KMT2A-CREBBP* (*CBP*)	
cryptic t(11;17)(q23.3;p13)	FAB M4 (t-AML)	*KMT2A-GAS7*	[262]
t(11;17)(q23.3;q12)[‡]	FAB M5 AML	*KMT2A-MLLT6* (*AF17*)	
t(11;17)(q23.3;q25)	t-AML, *de novo* AML, FAB M4, M5	*KMT2A-SEPT9*	[263]
t(11;19)(q23.3;p13.1)[‡]	FAB M5	*KMT2A-ELL*	[249]
t(11;19)(q23.3;p13.3)[‡]	FAB M5 (also B-lineage and T ALL)	*KMT2A-MLLT1*(*ENL*)	[249]
t(11;19)(q23.3;p13)	AML	*KMT2A-SH3GL1*(*EEN*)	[264]
t(11;19)(q23.3;q12 or q13)		Not known	
t(11;21)(q23.3;q13)		Not known	
t(11;22)(q23.3;q11)		*KMT2A-SEPT5* (*HCDCREL*)	

(Continued)

Table 3.4 (Continued)

Translocation or other rearrangement	Type of leukaemia	Molecular event	Reference
t(11;22)(q23.3;q13)	AML	*KMT2A-EP300* (*P300*)	[265]
t(X;11)(q13;q23.3)	FAB M4 or M5	*KMT2A-FOXO4* (*MLLT7*, *AFX1*)	
t(X;11)(q24;q23.3) – often complex‡	FAB M2	*KMT2A-SEPT6* (*KIAA0128*)	[266]
Tandem partial duplication of *KMT2A*, with or without trisomy 11	FAB M0, M1, M2, M4, M7	*KMT2A*-PTD	[267]

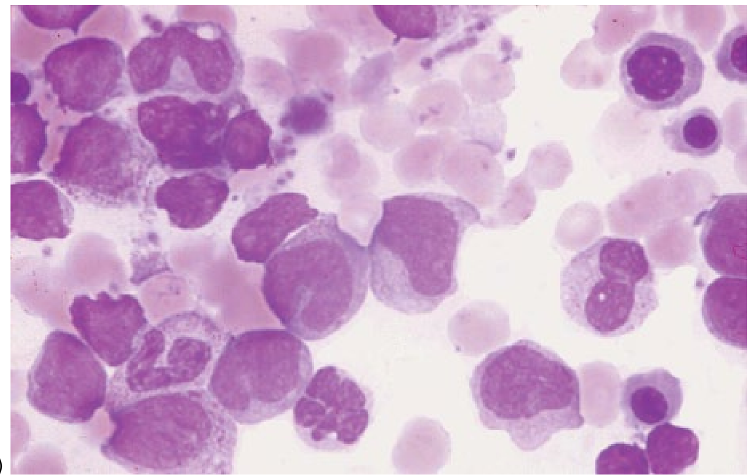
ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; FAB, French–American–British (classification); PTD, partial tandem duplication; t-AML, therapy-related AML.

* Some of these translocations are also associated with ALL or mixed phenotype 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.

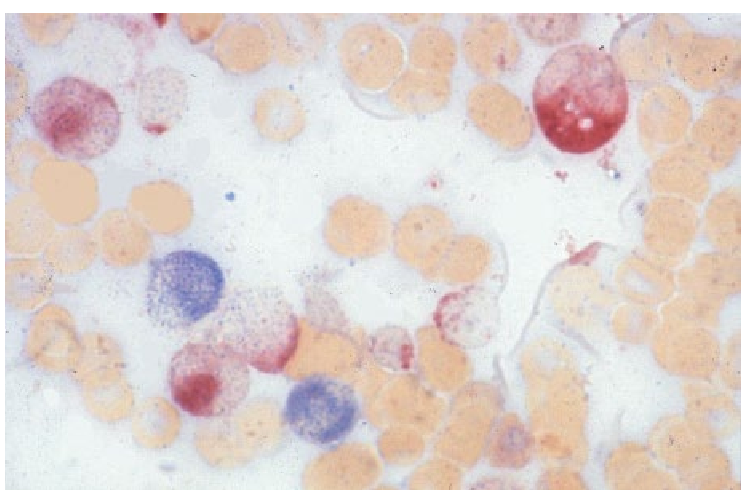
† Rearrangement of 11q23.3 may be detected by conventional cytogenetic analysis. Rearrangement of the *KMT2A* gene can be detected in a larger number of cases by FISH, RT-PCR or Southern blotting. FISH with a dual-colour, break-apart *KMT2A* probe is particularly useful, the fused signal being split 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.

‡ The eight rearrangements indicated, together with t(9;11)(p21.3;q23.3); *KMT2A-MLLT3*, represent 80% of *KMT2A* rearrangements in AML [268].

§ Cryptic gene fusion can also occur.



(a)



(b)

Fig. 3.36 BM film from a patient with FAB M4 AML associated with t(4;11)(q21;q23.3) 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.

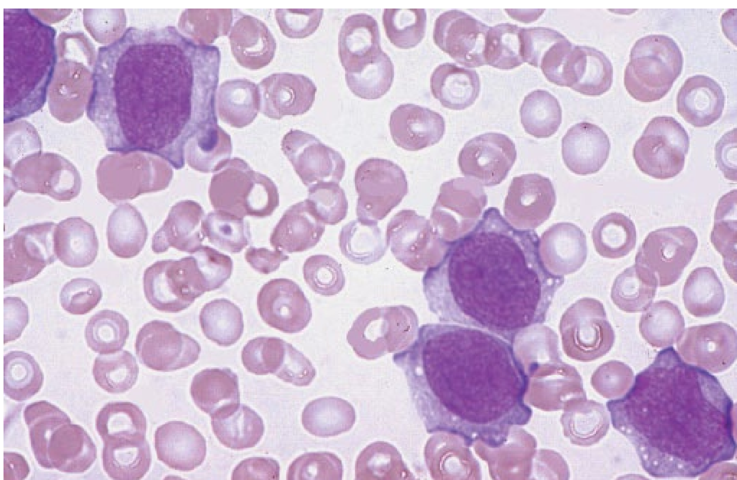


Fig. 3.37 BM film from a patient with acute monoblastic leukaemia (FAB M5a AML) associated with t(11;19)(q23.3;p13.3) showing monoblasts. MGG × 100.

Immunophenotype

The immunophenotype is generally similar to that of AML associated with t(9;11). NG2 expression has been demonstrated in cases with *KMT2A* rearrangement associated with t(11;17)(q23.3;q21) or t(11;19)(q23.3;p13) but is not specific for *KMT2A* rearrangement [241].

Cytogenetic and molecular genetic features

Cytogenetic analysis underestimates the frequency of cases with 11q23.3 rearrangement in comparison with molecular analysis [270]. FISH analysis, for example 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 [270]. The *KMT2A-AFDN* (previously *MLL-AF6* and *MLL-MLLT4*), *KMT2A-MLLT10* (previously *MLL-AF10*) and *KMT2A-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 [228]. In the case of *KMT2A-MLLT1*, RT-PCR detects more cases than are detected by conventional cytogenetic analysis [274]. Multiplex PCR can be used to detect the more common *KMT2A* rearrangements in a single reaction. RQ-PCR can be used to detect *KMT2A* duplication.

Acute myeloid leukaemia with t(6;9)(p23;q34.1); *DEK-NUP214*

Cases of leukaemia associated with t(6;9)(p23;q34.1) (Fig. 3.38) comprise less than 1% of cases of AML. They may be therapy-related or develop *de novo*. Therapy-related cases, categorized as t-AML, can follow exposure to either topoisomerase II-interactive drugs [22] or alkylating agents. Patients categorized as MDS with the same cytogenetic abnormality do not have an appreciably better prognosis [275] and an argument could therefore be made for regarding all as AML.

Clinical and haematological features

Patients having AML associated with t(6;9)(p23;q34.1) 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 [45,276,277]. A series of 34 patients entered into MRC trials had a 10-year survival of 26% [49].

Cases are usually FAB M2 AML, less often M4 and least often M1 [276–278]. Trilineage myelodysplasia is common [279,280], and overt AML may be preceded by MDS. There may be ring sideroblasts. The presenting WBC is generally low and there may be pancytopenia [7]. Blasts are usually granular [280]. The blast cell percentage

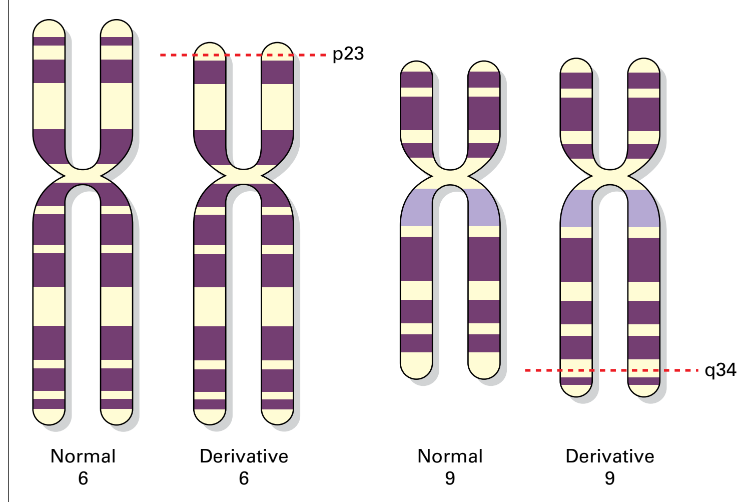


Fig. 3.38 Diagrammatic representation of t(6;9)(p23;q34.1) (modified from reference [2]).

may be relatively low (e.g. between 20% and 30%) [280]. Auer rods are reported as sometimes [280] or often [276,277] present. Bone marrow basophilia is common (Fig. 3.39) but not invariable and may also be present during a preceding myelodysplastic phase [276]. The peripheral blood basophil count is also often elevated [276]. Basophilic differentiation can be confirmed by metachromatic staining with toluidine blue (Fig. 3.39d). Some cases have also had increased bone marrow eosinophils. Peripheral blood eosinophilia can also be a feature [281] but is uncommon.

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 MPO, CD9, CD13, CD33, CD38, CD123 and HLA-DR [7,280] and often also of CD15, CD34 and CD117 [7]. In contrast to AML in general, TdT is often expressed [7,280]. Cases with monocytic differentiation may have expression of CD14 and CD64. Basophils express CD33, CD38 and CD123 [7].

Cytogenetic and molecular genetic features

The t(6;9)(p23;q34.1) rearrangement is shown in Fig. 3.40. 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 (previously known as *CAN*) at 9q34.1 to form a fusion gene, *DEK-NUP214* [282]. *NUP214* encodes a nucleoporin, that is a protein belonging to the nuclear pore complex [283], 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. t(6;9) is the sole cytogenetic abnormality in about 80% of patients, with trisomy 8 and trisomy 13 being the most frequent additional abnormalities [275].

A *FLT3*-ITD is found as a second genetic event in a large proportion of patients – up to 70% of adults but about 40% of children [173,178,280] whereas *FLT3*-TKD mutation is rare [178]. The prognosis is not significantly worsened by the presence of *FLT3*-ITD, although a real difference could be obscured by different treatment protocols [275,284].

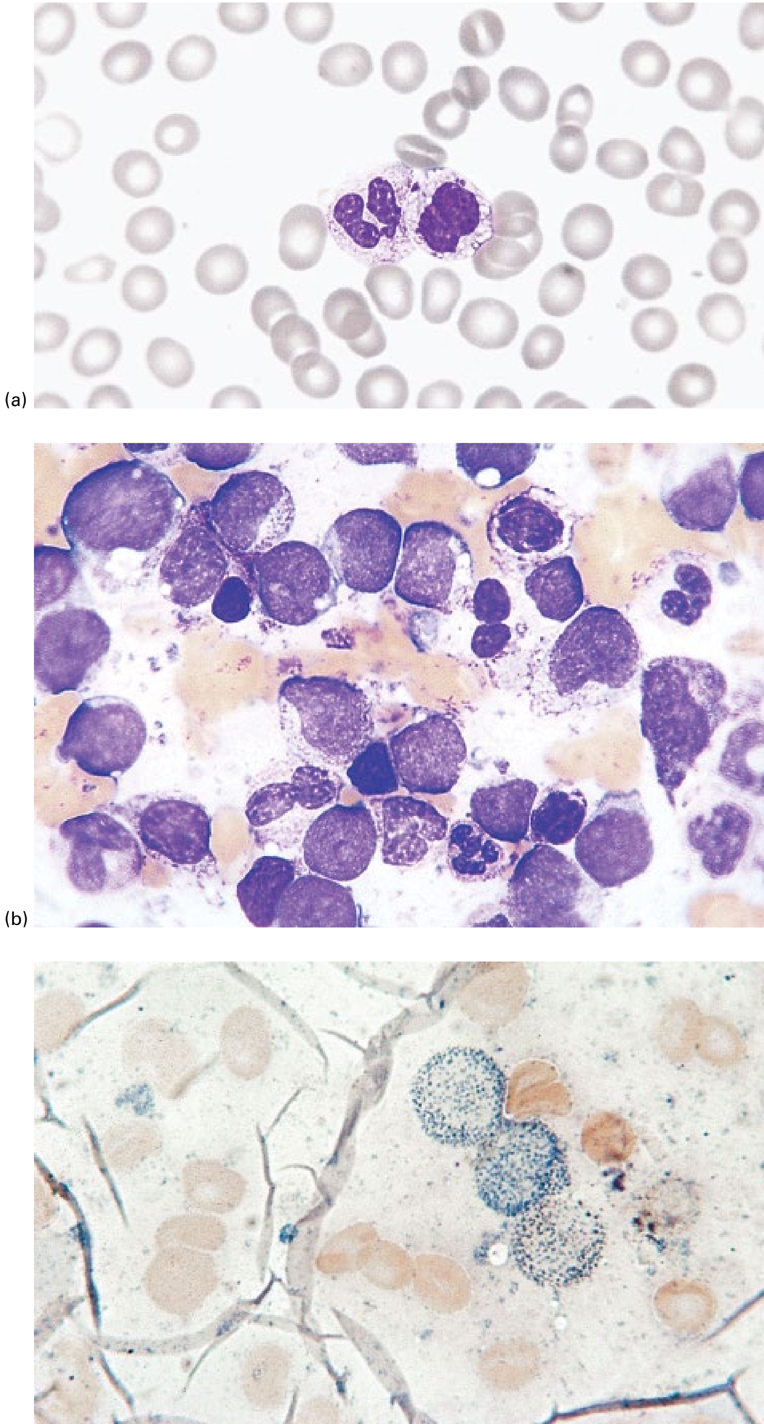


Fig. 3.39 PB and BM films from a patient with FAB M2Baso AML associated with t(6;9)(p23;q34.1). (a) PB film showing abnormal basophils. MGG × 100. (b) BM film showing neutrophilic and basophilic differentiation. MGG × 100. (c) BM film showing positive reaction for chloroacetate esterase (CAE). CAE × 100.

(d)

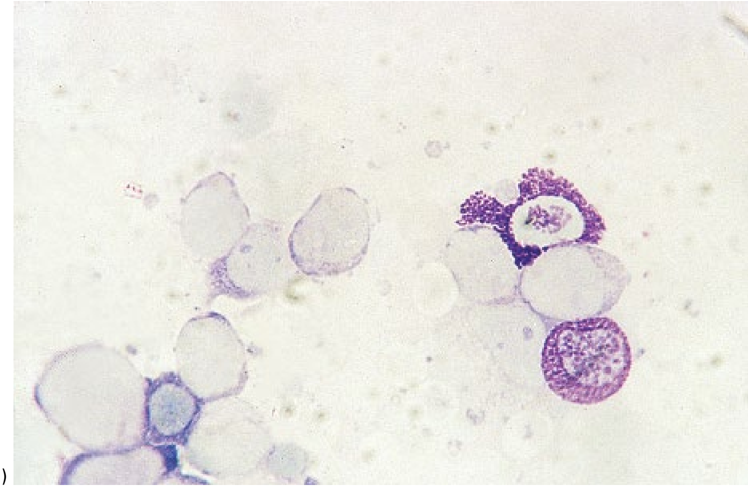


Fig. 3.39 (*Continued*) (d) BM film showing metachromatic staining with toluidine blue. Toluidine blue × 100. (With thanks to the late Dr David Swirsky.)

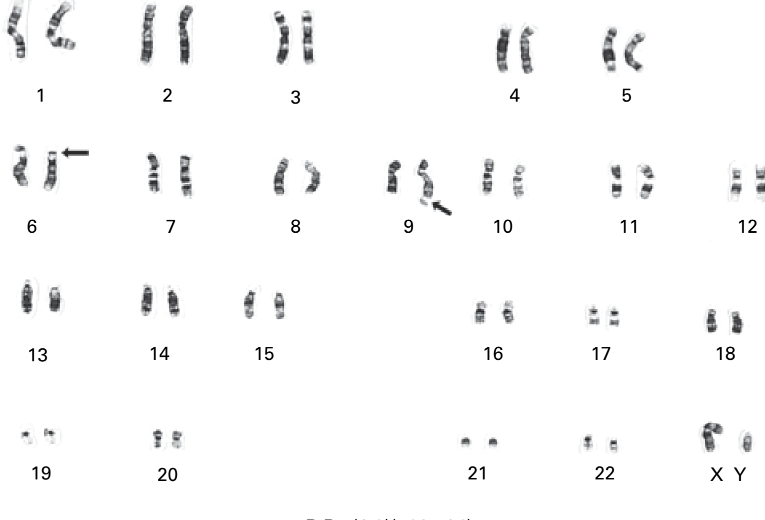


Fig. 3.40 Karyogram of the patient with FAB M2 (Baso) and t(6;9)(p23;q34.1) whose PB and BM films are shown in Fig. 3.39. (With thanks to the late Dr David Swirsky and Miss Julie Bungey, London.)

Acute myeloid leukaemia with inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *GATA2, MECOM*

Inversions (Fig. 3.41) and translocations (Fig. 3.42) with 3q21.3 and 3q26.2 breakpoints are found in no more than 2% of haematological neoplasms, including about 1% of cases of AML [18,285]. Both *de novo* and t-AML can be associated with 3q21.3q26.2 abnormalities [247,285]. Only the *de novo* cases fall into this WHO category. This group of disorders is sometimes referred to as the 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 myeloid leukaemia (CML). Prognosis in patients with these genetic abnormalities is unrelated to the blast percentage, the median survival being equally poor in patients classified as AML and in those classified as MDS [286].

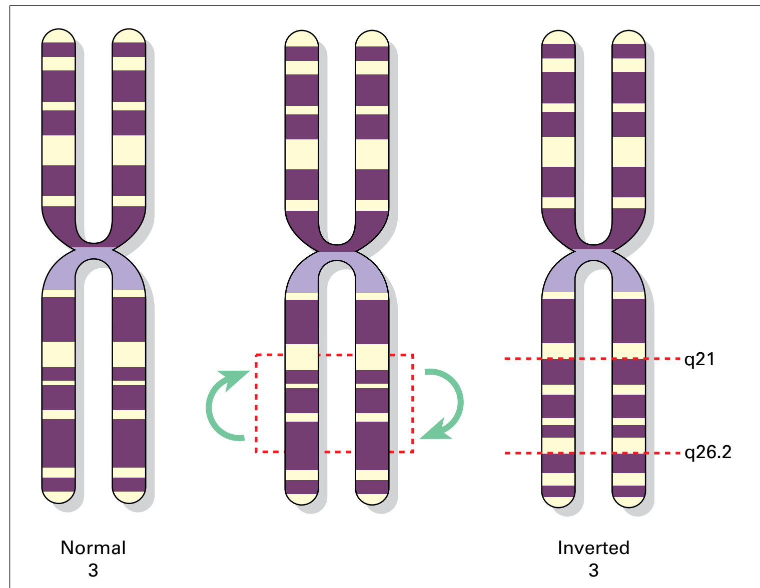


Fig. 3.41 A diagrammatic representation of inv(3)(q21.3q26.2); this is an example of a paracentric inversion (modified from reference [2]).

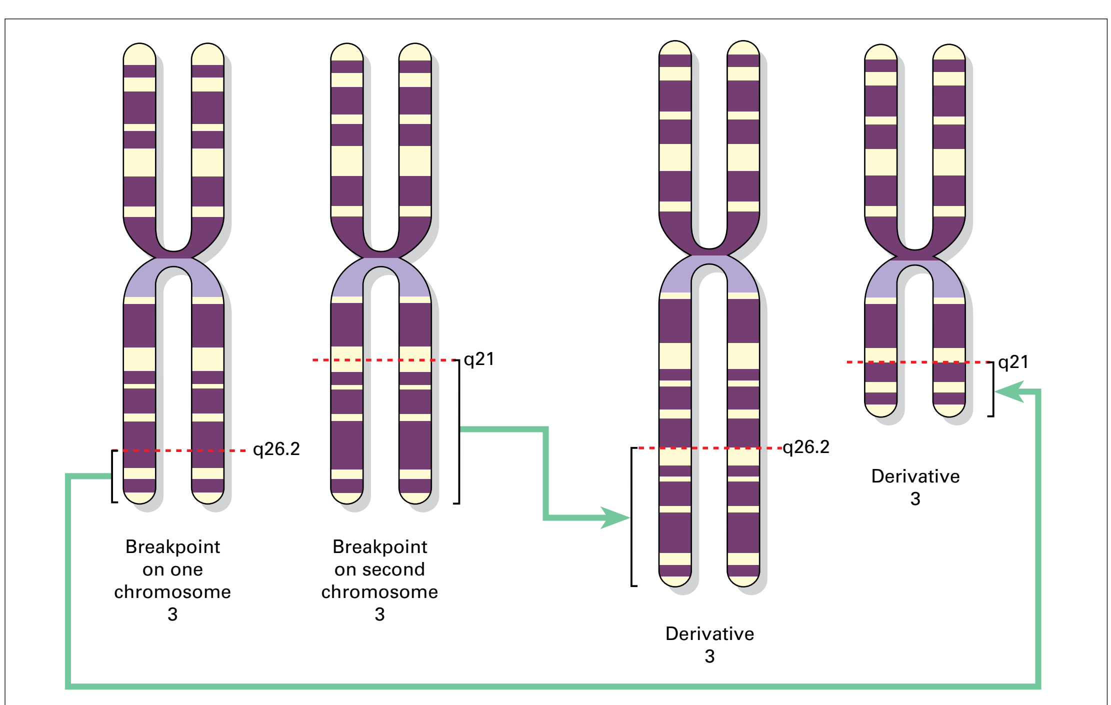


Fig. 3.42 A diagrammatic representation of t(3;3)(q21.3;q26.2).

Clinical and haematological features

Cases occur throughout adult life with the mean age being somewhat younger than that of AML in general [287]. There is a female preponderance [287]. 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 [288,289]. The AML may be of any FAB category with the exception of M3 AML, but cases of M7 AML are over-represented [206,285]. This subtype 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. Dysgranulopoiesis is an independent poor prognostic feature [286]. There may be giant or hypogranular platelets [285,290] and bare megakaryocyte nuclei in the peripheral blood [7]. Auer rods are not a feature. Eosinophils and basophils are sometimes increased. Mast cells may be increased [7]. The bone marrow may be hypocellular [7]. 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 3.43 and 3.44). 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 [45,48,287]. The 5-year survival is low [19,44,45]. A series of 65 patients entered into MRC trials had a 10-year survival of only 3% [49]. Poor prognosis is not confined to patients with monosomy 7 or a complex karyotype [287].

Immunophenotype

A study of 35 patients found that more than 90% of cases showed expression of CD34, CD13 and CD33 [291]. Other antigens expressed and the percentage of positive cases were: CD117 (87%), CD7 (61%), CD123 (60%), CD11b (50%), CD4 (39%), MPO (37%), CD14 (35%), CD65 (33%), CD56 (20%) and CD41/61 (16%) [291]. Terminal deoxynucleotidyl transferase was uniformly negative. HLA-DR and CD38 are usually positive [7].

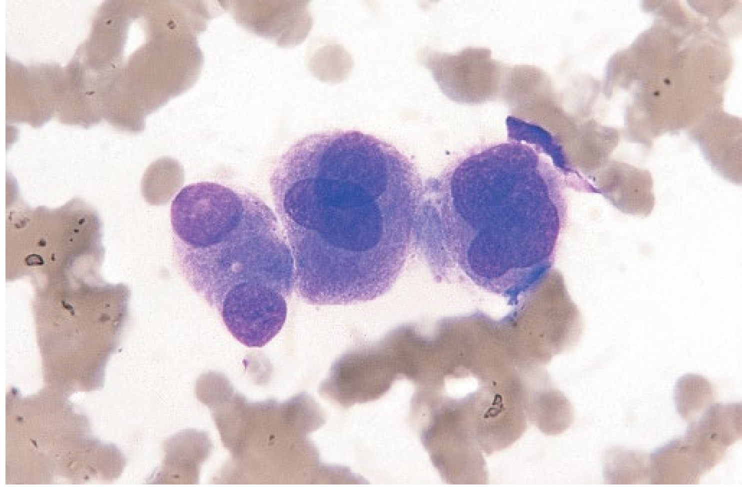


Fig. 3.43 BM film from a patient with inv(3)(q21.3q26.2) showing increased numbers of dysplastic megakaryocytes. MGG × 100. (With thanks to Dr Guy Lucas, Manchester.)

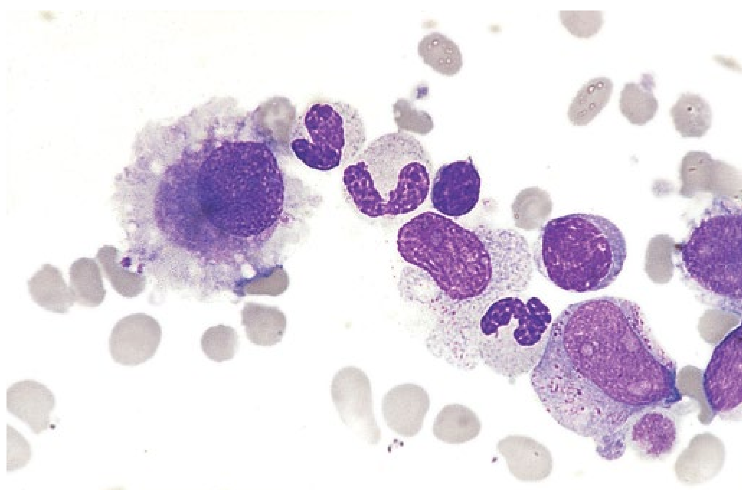


Fig. 3.44 BM film from a patient with t(3;3)(q21.3;q26.2) showing hypogranular neutrophils and a hypolobulated megakaryocyte. MGG × 100. (With thanks to colleagues in the United Kingdom Cancer Cytogenetics Study Group [285].)

Cytogenetic and molecular genetic features

The most common cytogenetic abnormality is inv(3)(q21.3q26.2) (Fig. 3.45), with about a fifth of cases having t(3;3)(q21.3;q26.2) (Fig. 3.46) and occasional patients having ins(2;3)(p21;q21q26), ins(3;3)(q23–26;q21q26), ins(5;3)(q14;q21q26) or ins(6;3)(q23;q21q26) [287,292].

The commonest secondary cytogenetic abnormalities are monosomy 7 (which occurs in as many as half of patients), trisomy 8, a complex karyotype and del(5q) [244,285,287]. Prognosis is worse if there is a monosomy 7, a complex karyotype or a monosomal karyotype [7,286].

The molecular mechanism of leukaemogenesis is dysregulation of *MECOM* at 3q26.2 by repositioning of a *GATA2* enhancer, which at the same time produces *GATA2* haploinsufficiency [7]. When this genetic abnormality is present the case is assigned to this AML category, regardless of the precise cytogenetic abnormality. Occasionally there is rearrangement and truncation of *MECOM* [293] but this is not usual [294]. *MECOM* 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 [294]; 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 [295]. *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 [90]. *NRAS* mutations were particularly common in patients with t(3;5) in one study, being found in three of eight patients [89], but in another study a mutation was found in none of 14 patients [90]. *FLT3*-ITD is less common than in AML in general, being only 13% in one series [287]. Other genes that are mutated in more than 10% of patients include *SF3B1*, *PTPN11*, *KRAS*, *GATA2* and *RUNX1* [7].

This and other rearrangements of 3q26.2 can be detected by dual-colour, break-apart FISH [292]. 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) [285,296], t(3;5)(q21;q31) [285,297], t(3;6)(q21;p21) [298], t(3;12)(q26;p13)

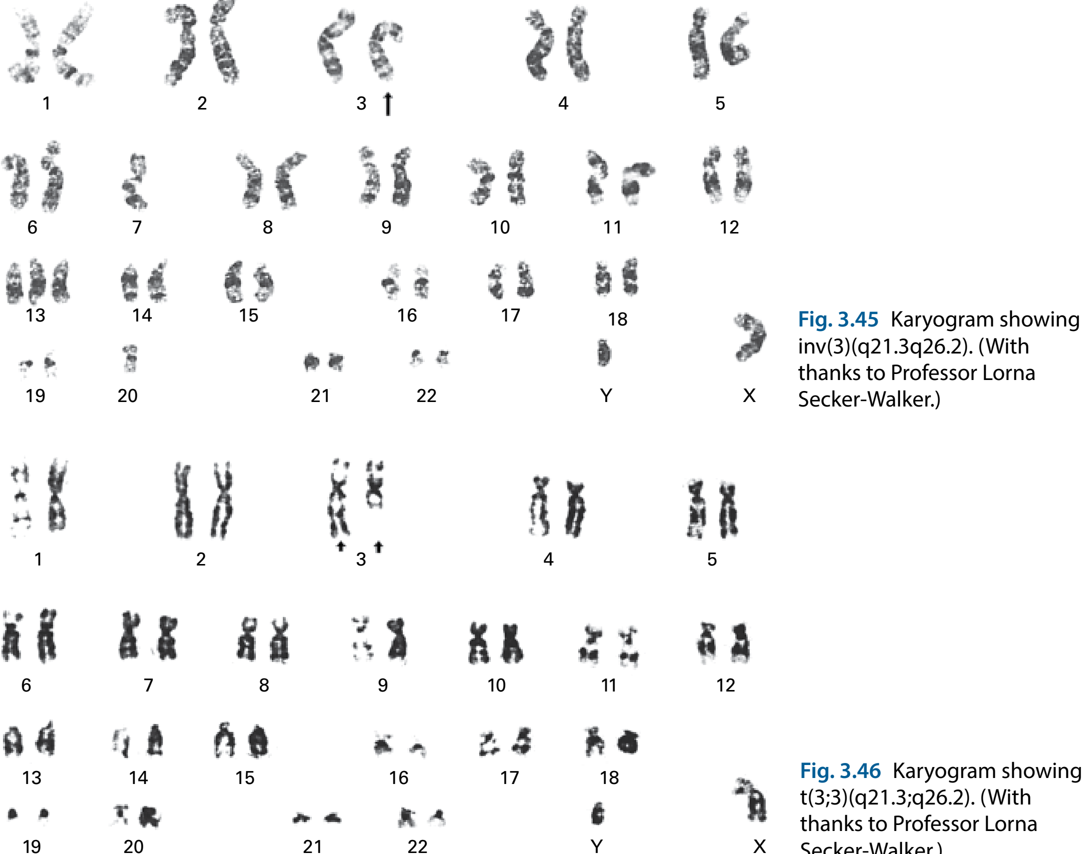


Fig. 3.45 Karyogram showing inv(3)(q21.3q26.2). (With thanks to Professor Lorna Secker-Walker.)

Fig. 3.46 Karyogram showing t(3;3)(q21.3;q26.2). (With thanks to Professor Lorna Secker-Walker.)

with *ETV6-MECOM* [285,299], t(3;21)(q26.2;q22) with *RUNX1-MECOM* (which is usually t-AML) [285,300,301] and del(3)(q12q21) [290].

Acute leukaemia (megakaryoblastic) with t(1;22)(p13.3;q13.1); *RBM15-MKL1*

AML associated with t(1;22)(p13.3;q13.1) 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 [302].

Clinical and haematological features

The median age of reported cases is under 6 months [206,303,304]. Identical twins showing concordance for this subtype of AML have been described, suggesting a possible intrauterine origin of the leukaemia [305], and congenital cases have been reported [269,306,307]. There may be a female preponderance [302]. Hepatomegaly and splenomegaly are common and may be marked. The bone marrow blast percentage is low, with a series of 10 patients having a median of 22% blasts [304]; in 4 of the 10 patients, blasts were less than 20%. The blast cells may show characteristic cytological features of megakaryoblasts (Fig. 3.47) or may appear morphologically undifferentiated. An aleukaemic case with less than 5% bone marrow blast cells but with hepatic tumours has been reported [306]. Haemophagocytosis by leukaemic blast cells has been reported [308]. There is dysmegakaryopoiesis with some micromegakaryocytes [206]. Bone marrow fibrosis, both reticulin and collagen, can occur (Fig. 3.48). Prognosis is intermediate with a reported remission rate of 55% and 4-year survival of approximately 30% [48].

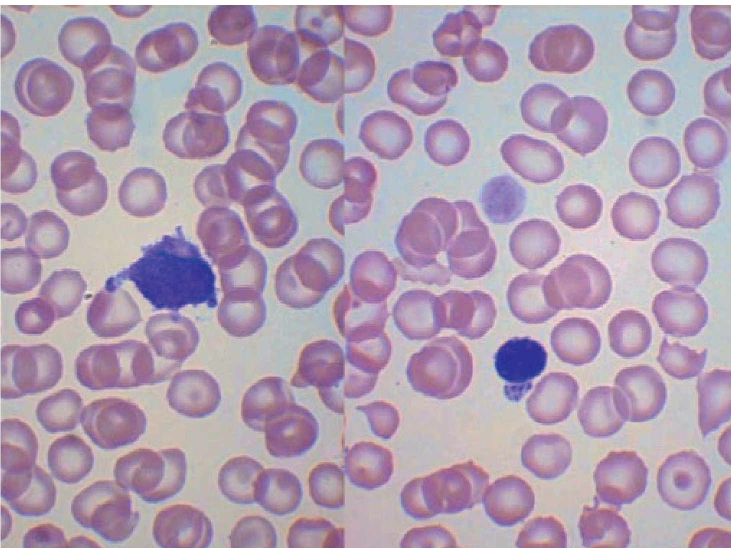


Fig. 3.47 Composite photograph of PB film of an infant with acute megakaryoblastic leukaemia associated with t(1;22)(p13.3;q13.1) showing a giant platelet and two megakaryoblasts with granular blebbed cytoplasm. MGG × 100.

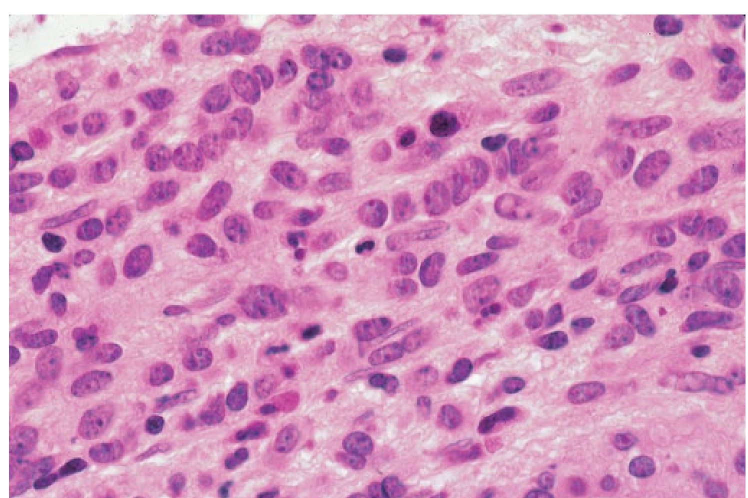


Fig. 3.48 Trephine biopsy section from a patient with FAB M7 AML associated with t(1;22)(p13.3;q13.1). Haematoxylin and eosin (H&E) × 100. (With thanks to Dr Richard D. Brunning, Minnesota.)

Immunophenotype

There is expression of platelet glycoproteins such as CD41 and CD61 (less often CD42b) [7]. CD13 and CD33 may be expressed but CD34 and HLA-DR are usually negative [7]. CD36 may be expressed [309]. CD11b and CD15 are usually negative [309]. CD45 expression may be weak, and initial misdiagnosis as a non-haemopoietic tumour has been common among reported cases [244].

Cytogenetic and molecular genetic features

Cytogenetic analysis shows t(1;22)(p13.3;q13.1) (Fig. 3.49). 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 [48,244,303,310,311]. The molecular mechanism is formation of an *RBM15-MKL1* fusion gene (previously known as *OTT-MAL*) on chromosome 1 [312,313]; the reciprocal *MKL1-RBM15* fusion gene is not transcribed in

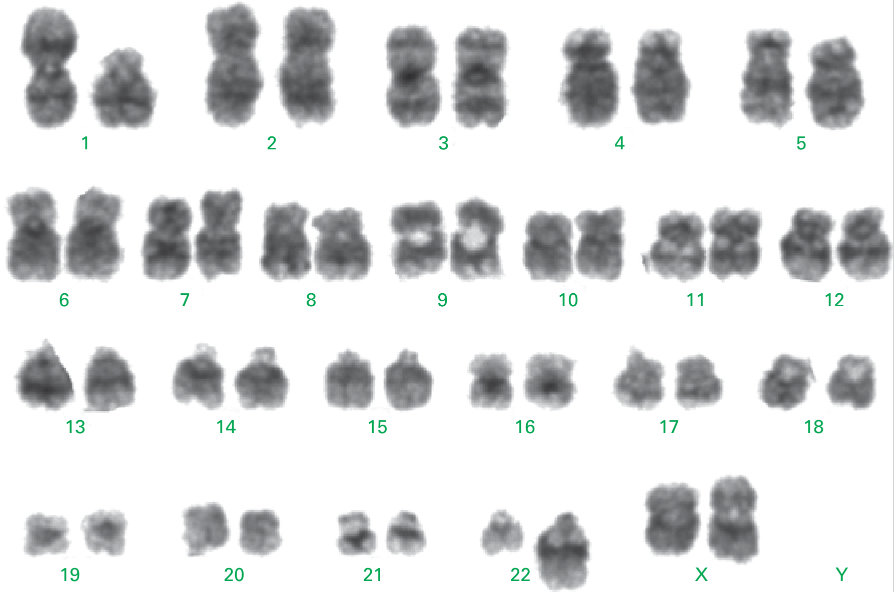


Fig. 3.49 Karyogram showing t(1;22)(p13.3;q13.1). (With thanks to Steve Chatters and Dr Helena Kempski, London.)

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 [206,302].

Acute myeloid leukaemia with t(9;22)(q34.1;q11.2); *BCR-ABL1*

Patients with AML and t(9;22)(q34.1;q11.2); *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 [314]. Most cases occur *de novo* but therapy-related cases are recognized and are classified as t-AML [315,316]. The prognosis of therapy-related cases is, if anything, even worse than that of *de novo* cases [316]. In the 2016 revision of the WHO classification, this is recognized as a provisional entity [7].

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 [48,317]. Five-year survival was negligible in one series of patients [317] but a group of 47 MRC trial patients had a 10-year survival of 16% [49]. Even imatinib-treated patients have a poor prognosis due to failure to achieve a complete remission and short remission duration [317]. *BCR-ABL1*-positive AML should be distinguished from blast crisis of a previously undiagnosed CML. Patients with AML are less likely to have splenomegaly although massive splenomegaly can occur [317]. The basophil count is much less often elevated [317]. 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 [318]. Such cases are excluded from this provisional WHO category.

Clinical and haematological features

Clinical features differ from those of Ph-negative AML in that hepatomegaly and splenomegaly are more common [319]; nevertheless, only a minority of patients have splenomegaly [317]. The basophil count can be increased but in the majority of patients it is not [317]. Only a minority of patients have an increase of bone marrow basophils [317]. Cases are usually FAB M0 or M1

and less often M2, M4, M6 or M7. MPO activity may be weak or absent [317,320]. Auer rods are uncommon. Erythrophagocytosis by leukaemic blast cells was reported in one patient [321].

Immunophenotype

There are no specific immunophenotypic features. There is usually expression of CD13, CD33 and CD34 but not TdT [317]. Lymphoid antigens such as CD7 and CD19 are sometimes expressed.

Cytogenetic and molecular genetic features

The t(9;22)(q34.1;q11.2) rearrangement (Fig. 3.50) is the same as that seen in CML (see page 375). Variant translocations also occur, for example t(9;11;22)(q34;q12;q11.2) and t(9;22;21)(q34; q11;p11) [317]. The majority of patients have secondary clonal abnormalities or clonal evolution [317]. The commonest associated chromosomal aberrations are monosomy 7, trisomy 8 and 19, and duplication of the Ph chromosome, der(22)t(9;22) [244]. Other secondary abnormalities recognized in blast crisis of CML can also occur, for example i(17)(q10) and inv(3)(q21.3q26.2) [317]. 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 [317].

A number of FISH strategies are available for detection of t(9;22) (see Fig. 4.6). The BCR-ABL1 protein can also be detected by flow cytometry using a commercially available bead assay [322].

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 CML, rather than in the minor breakpoint cluster region (m-BCR), characteristic of ALL. Other molecular genetic features differ from those of myeloid blast crisis of CML but have similarities to lymphoid blast crisis and Ph-positive ALL [323]. Loss of the *IKZF1* or *CDKN2A* and *CDKN2B* genes, as seen in Ph-positive ALL, can occur, as can cryptic deletions within the IGH and TRG loci [323]. *NPM1* mutation can occur as a second abnormality in Ph-positive AML whereas *ABL1* mutation can occur in blast crisis of CML [324].

Acute myeloid leukaemia with *NPM1* mutation

Mutations of *NPM1* at 5q35 have been detected in around a third of patients with *de novo* AML, and less frequently in t-AML [325–328]. In those with a normal karyotype the frequency in different series of patients ranges from 46% to 62% [329]. The *NPM1* gene encodes nucleophosmin,

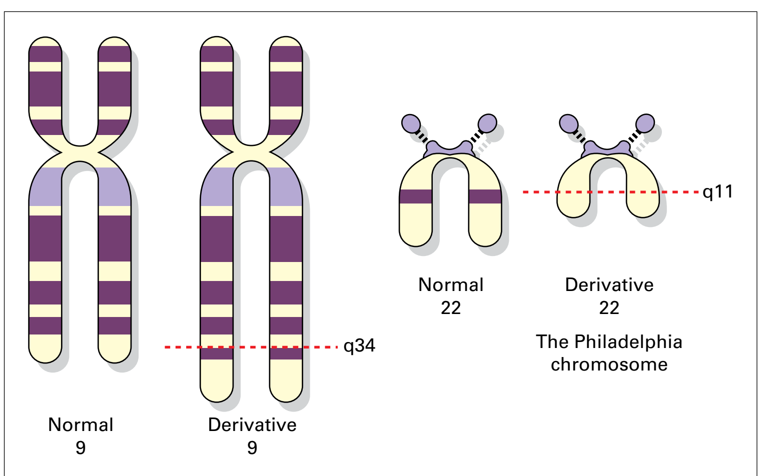


Fig. 3.50 Diagrammatic representation of t(9;22)(q34.1;q11.2). (Modified from reference [2]).

a phosphoprotein that shuttles between the nucleus and cytoplasm, and the mutation leads to cytoplasmic rather than nuclear (mainly nucleolar) localization of the protein. In the absence of the recurrent genetic abnormalities described above, *de novo NPM1*-mutated AML is a specific entity in the 2016 revision of the WHO classification. Cases with an abnormal karyotype appear to have the same disease characteristics and the same prognosis as those with a normal karyotype [330,331], and the presence of a karyotypic abnormality is no longer a criterion for exclusion from this category. Although *NPM1* mutation defines this category it is not necessarily the first leukaemogenic mutation [7].

Clinical and haematological features

These mutations are more common in women than in men [326,327,332] 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 [325,332,333] and with gingival hyperplasia [326], lymphadenopathy [326] and skin infiltration. Five-year survival is about 60% [326]. The WBC [326,332–334], bone marrow blast percentage [327] and platelet count [327] 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. 3.51) has been observed [335,336], and in one comparison of 17 patients with *NPM1* mutation and 49 without, the presence of more than 10% of blasts with cup-like nuclei was found to be highly specific with a 30% sensitivity [337]. The association is particularly with coexisting *NPM1* mutation and *FLT3*-ITD [338]. Cup-shaped nuclei are not specific for *NPM1* mutation; in one patient this abnormality was associated with AML with t(9;22) [339], and it is also occasionally observed in ALL, in one patient again being associated with *BCR-ABL1* [340]. There is a high frequency of *IDH1* or *IDH2* mutation among cases with cup-shaped nuclei and *NPM1* mutation [341]. Pseudo-Chédiak–Higashi granules have been reported [309]. *NPM1* mutation may not be the initial leukaemogenic event. In one study, mutation of *DNMT3A* appeared to have preceded *NPM1* mutation [342].

Immunophenotype

The immunophenotype may be that of an immature myeloid cell or there may be features of monocytic differentiation [7]. There is expression of CD13, CD33 and MPO and often of

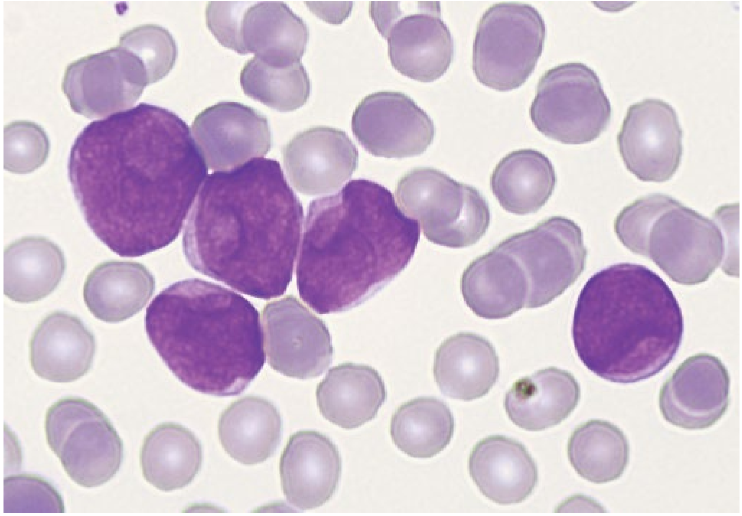


Fig. 3.51 PB film from a patient with AML with a normal karyotype who had both an *NPM1* mutation and *FLT3*-ITD showing blast cells with cup-shaped nuclei. MGG × 100. (With thanks to Dr Safia Jalal and Dr Mike Leach, Glasgow.)

CD14, CD11b and CD68R [7]. CD33 expression is usually strong, suggesting the possibility of benefit from immunotherapy directed at this antigen [343]. CD15, CD117 and CD123 are usually positive [344]. CD2 and CD14 expression have been found to be less often positive than in *NPM1* wild-type AML, with CD4 being more often positive [345]. The stem cell markers, CD34 and CD133, are characteristically negative [332] but the stem cell marker CD110 is positive in most cases [344]. HLA-DR is expressed in about two-thirds of patients, and CD7 is expressed in a minority. CD56 expression has been found to correlate with *FLT3*-ITD but not with mutated *NPM1* [345]. Cytoplasmic rather than nucleolar expression of NPM1, detectable by immunohistochemistry [346] or flow cytometry [347], acts as a surrogate marker for an *NPM1* mutation, with C23, a monoclonal antibody to nucleolin, sometime being used as a control. However, occasional mutations are associated with nucleolar localization or nucleolar plus cytoplasmic [348], and the detection of cytoplasmic positivity can be affected by the fixative used. Distribution of PML protein is also abnormal, with nuclear bodies being of variable size and staining intensity with a background microspeckled pattern [166].

Cytogenetic and molecular genetic features

The mutations (small duplications or insertions) all lead to a frameshift in the region of the gene that encodes the C-terminal protein. Mutations can be detected by multiplex PCR-based fragment length analysis [349]. The karyotype is characteristically but not necessarily normal. In a large series of patients, karyotypic abnormalities, including +8, +4, −Y, del(9q) and +21, were found in 15% of patients [330]. Rare examples have been reported in which an *NPM1* mutation coexisted with t(8;21), t(9;22), t(15;17) or inv(16) [327], and such cases are assigned to the relevant cytogenetic category. However, in general *NPM1* mutation and recurrent chromosomal rearrangements are mutually exclusive, none being found in a series of more than 2000 patients with AML [350]. Patients with a normal karyotype at diagnosis may have an abnormality at relapse, and those with an abnormality at diagnosis may show a different abnormality [330]. A chromosomal abnormality may be present in only a proportion of leukaemic cells [330]. It thus appears that cytogenetic abnormalities are a secondary event. In about 10% of patients an *NPM1* mutation is absent at relapse [351]. The gene expression profile is distinctive [334,352].

NPM1 is not the founder mutation, this generally being preceded by mutation in *DNMT3A*, *IDH1* or *NRAS* [353]. There is a high frequency of associated *FLT3*-ITD, which appears to be a later event [327]. *FLT3*-ITD is associated with worse overall survival in *NPM1*-mutated cases with a normal karyotype [331,354] regardless of the mutant allele level [354]. *NPM1*-mutated AML without *FLT3*-ITD shows an improved outcome when ATRA is added to intensive chemotherapy [355]. There is a significant association between *NPM1* and *TET2* mutations [356]. *DNMT3A*, *KRAS*, *NRAS*, *IDH1* and *IDH2* mutations may also be present [7]. There is an inverse association with *CEBPA* mutations, *NRAS* mutations, *KMT2A* partial tandem duplication (*KMT2A*-PTD), t(8;21), t(15;17), inv(16) and complex karyotypes [327,333,334]. In one study prognosis did not differ between patients with a normal karyotype with or without an *NPM1* mutation [333]. 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 [326,332], longer event-free survival [326,327,332,334] and longer overall survival [326,327,334]. Coexisting *DNMT3A* and *NPM1* mutation is associated with a good prognosis but when there is also *FLT3*-ITD the prognosis is very adverse [353]. Patients with an *NPM1* mutation but without *FLT3*-ITD mutation or adverse cytogenetic abnormalities could thus reasonably be assigned to the good prognosis category of AML. Favourable prognosis has also been related to *NPM1* mutation in conjunction with *IDH1* or *IDH2* mutation [239]. Survival in *NMP1*-mutated AML may be better with high-dose daunorubicin

rather than standard dose [239]. MRD can be monitored by RQ-PCR; three sets of primers cover 90% of mutations. MRD detected by RQ-PCR after the second cycle of chemotherapy is associated with an adverse prognosis [357].

Acute myeloid leukaemia with biallelic *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) [328,358]. Among patients with a normal karyotype, the prevalence in a large series of patients was 8–9% [359]; in children with a normal karyotype the prevalence was 17%, most of these being biallelic mutations [360]. Biallelic mutation is associated with different disease characteristics and is associated with a better prognosis than monoallelic mutation. The 2016 revision of the WHO classification therefore requires biallelic mutation for inclusion in this category [7]. Absence of the recurrent cytogenetic abnormalities that define categories already described is also required. In children and young adults the prevalence of biallelic mutation is 4–9% [7].

Germline heterozygous mutation of this gene is associated with adult-onset AML in a rare familial syndrome [361].

Clinical and haematological features

There is sometimes lymphadenopathy, and myeloid sarcoma can occur [7] but extramedullary disease is not common. *CEBPA* mutations have been associated particularly with FAB M1 and M2 AML [362,363] but have also been observed in M0, M4, M5 and M6 categories. Bone marrow eosinophil precursors may be increased [364]. Multilineage dysplasia was observed in 26% of a series of 108 patients and was not of adverse prognostic significance [362].

Immunophenotype

There is expression of myeloid antigens such as CD13, CD33, CD65, and CD15 [7,365,366]. CD34 and HLA-DR are usually expressed and CD7 is expressed in half to three-quarters of patients [7]. CD11b, CD14, CD56 and CD64 are generally not expressed [365]. In comparison with monoallelic mutation, cases with biallelic mutation are more likely to express HLA-DR, CD7 and CD15, and less likely to express CD56 [366].

Cytogenetic and molecular genetic features

The karyotype is usually normal (74%) [362] but *CEBPA* mutations can be associated with miscellaneous cytogenetic abnormalities. Chromosomal abnormalities most often observed are −7 and +8 with only 5–6% of patients having an adverse karyotypic abnormality, specifically −7 [362]. There is also an association with del(9q) [367] and its presence does not exclude this diagnosis. However, in the 2016 revision of the WHO classification, the presence of other myelodysplasia-related cytogenetic abnormalities does exclude this diagnosis [7].

Mutations may be either an N-terminal dominant negative frameshift loss-of-function mutation or a C-terminal mutation that reduces DNA binding [367]. In a series of children with *CEBPA* mutation, 80% had biallelic mutations [360]. In a series of adolescents and adults, 50% of patients had biallelic mutations (including 11% who were homozygous) [362]. In about 9% of patients, biallelic mutation is the result of a germline mutation plus a somatic mutation [363]. *FLT3*-ITD has been reported in 5–9% of patients with a biallelic *CEBPA* mutation [7,362] and *FLT3*-TKD mutation in about 4% [362]; *FLT3*-ITD is more common in patients with monoallelic *CEBPA* mutation [368]. In one series of patients, approaching 4% of patients with AML and a normal karyotype had mutation of both *CEBPA* and *NPM1* [329]; however, in another series *NPM1* mutation was found in 14% of patients with *CEBPA* mutation but was seen only in patients with a monoallelic *CEBPA* mutation [362]; the association only with monoallelic *CEBPA* mutation was similarly observed in another series of patients [368]. Other genes that may be mutated include *IDH1* and *IDH2* (together 15% of cases), *RUNX1* (10% of cases) [362] and *CSF3R* (4/14 cases) [369]; *KMT2A*-PTD is found in about 4% of patients [362]. There is a strong association of biallelic *CEBPA* mutation with *GATA2* mutation, this being seen

in 13 of 33 instances in one series of patients [370]. In a detailed investigation of 95 patients with biallelic mutation the frequency of mutations in other genes was: *TET2* (34%), *GATA2* (21%), *WT1* (13.7%), *DNMT3A* (9.6%), *ASXL1* (9.5%), *NRAS* (8.4%), *KRAS* (3.2%), *IDH1* or *IDH2* (6.3%), *FLT3*-ITD (6.3%), *FLT*-TK domain (2.1%), *NPM1* (2.1%) and *RUNX1* (1/94) [363]. *TET2* mutation is prognostically adverse in biallelic mutated cases, whereas *GATA2* mutation may be prognostically favourable [363]. *CEBPA* biallelic mutation may be a second event following mutation of *TET2* or *DNMT3A* [363].

Biallelic *CEBPA* mutations are associated with a good prognosis [360,362,371,372]; the influence, if any, of *FLT3*-ITD in patients with *CEBPA* mutation is not clear [362,371,372] but probably there is no influence [362,372]. Patients with a biallelic *CEBPA* mutation can 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 [358].

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 [373]. The gene expression profile of biallelic mutated cases is specific and differs from that of cases with a monoallelic mutation [7]. Multiplex PCR-based fragment length analysis, with a false-negative rate of less than 1%, is suitable for routine diagnostic use [359].

Acute myeloid leukaemia with *RUNX1* mutation

RUNX1 mutation is found in 4–16% of patients with AML, with a higher frequency in older patients [7]. Such cases are assigned to a provisional category in the 2016 revision of the WHO classification as long as the previously described category-defining cytogenetic and molecular abnormalities are absent, the condition is not therapy related and myelodysplasia-related cytogenetic abnormalities are absent [7]. AML with mutated *RUNX1* can occur in patients with Fanconi anaemia or severe congenital neutropenia. Prognosis has been adverse in some studies.

Clinical and haematological features

RUNX1-mutated AML has a higher prevalence in older adults. It is associated with multiple FAB subtypes of AML but is most frequent among cases with M0 cytological features.

Immunophenotype

There is usually expression of CD13, CD34 and HLA-DR with variable expression of CD33, monocytic markers and MPO [7].

Cytogenetic and molecular genetic features

The karyotype may be normal or show abnormalities such as trisomy 8 or trisomy 13.

In some instances the mutation is germline. Other genes that may be mutated include *ASXL1*, *KMT2A* (PTD), *FLT3* (ITD), *IDH1* and *IDH2*. If an *NPM1* mutation or biallelic *CEBPA* mutation is present, assignment should be to that category [7].

Acute myeloid leukaemia with myelodysplasia-related changes

Most studies have found multilineage dysplasia to have an adverse prognostic significance in patients with AML [374–378]. 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 karyotypes [376]. 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 [379] (Fig. 3.52). These criteria for AML with myelodysplasia-related changes (AML-MRC) were 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 (NOS) [380]. 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) [380]. However, the prognosis of cases assigned to this category is not necessarily homogeneous; for example, children with monosomy 7 had a significantly poorer outcome than children with del(7q) [381]. A Chinese study found that patients with relevant cytogenetic abnormalities or a previous history of MDS or a myelodysplastic/myeloproliferative neoplasm (MDS/MPN) had a worse prognosis than AML, NOS but those with only multilineage dysplasia did not [382]. In a group of 443 childhood cases, event-free survival was found to be worse than that of AML, NOS but overall and relapse-free survival were not significantly worse [383]. In the 2016 revision of the WHO classification, further amendments to the criteria were made, specifically: (i) multilineage dysplasia in a patient with *NPM1* mutated or with a biallelic mutation of *CEBPA* does not lead to the case being assigned to AML-MRC; and (ii) the presence of del(9q) is no longer regarded as a myelodysplasia-related change [384].

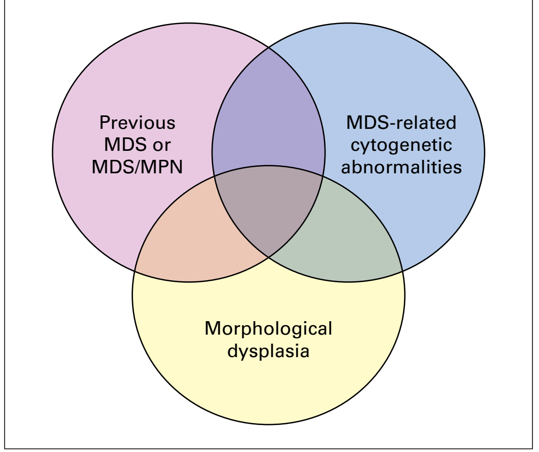


Fig. 3.52 A diagram illustrating that cases may qualify to be classified as myelodysplasia-related AML by meeting one, two or three criteria. MDS/MPN, myelodysplastic/myeloproliferative neoplasm.

Clinical and haematological features

Patients tend to be elderly and may have had previous MDS or 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 3.53–3.55). Prognosis

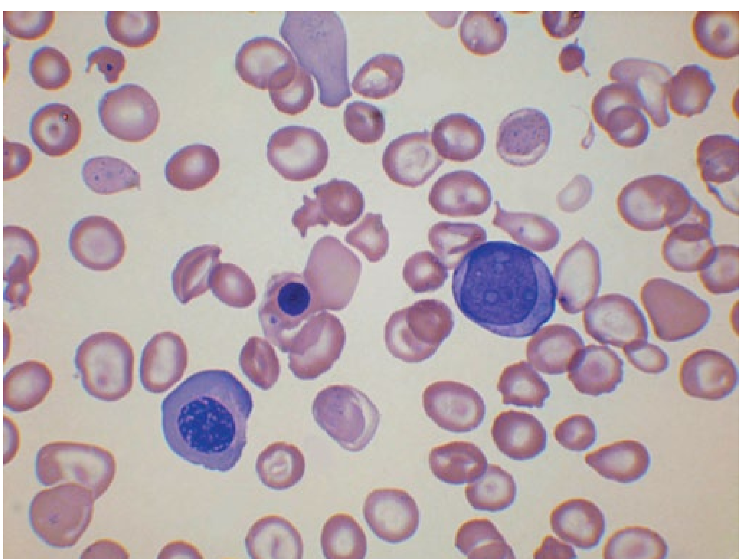


Fig. 3.53 PB film from a patient with myelodysplasia-related AML showing a blast cell and marked anisopoikilocytosis, basophilic stippling, polychromasia and nucleated red blood cells (one of which is megaloblastic). MGG × 100.

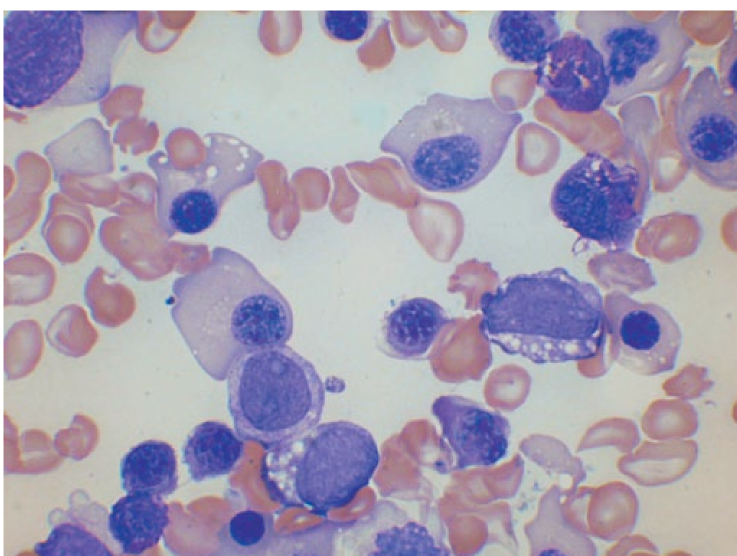


Fig. 3.54 BM film from a patient with myelodysplasia-related AML (same patient as Fig. 3.53) showing two vacuolated blast cells and dyserythropoiesis, including the presence of numerous megaloblasts. MGG × 100.

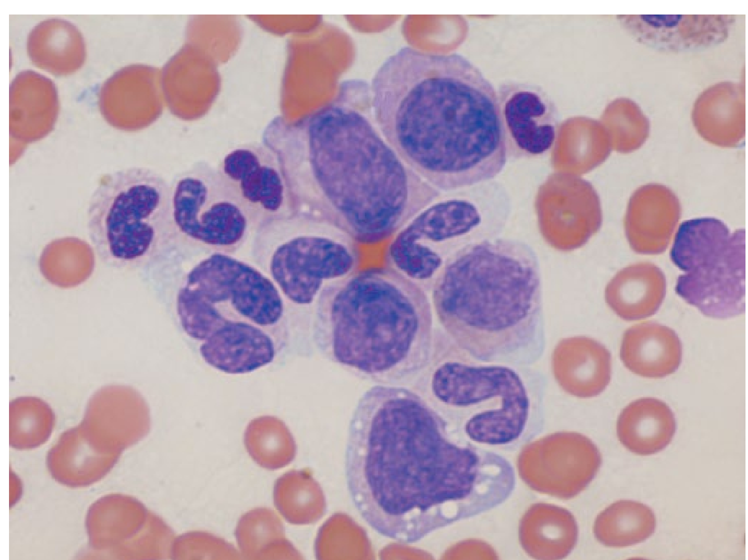


Fig. 3.55 BM film from a patient with myelodysplasia-related AML showing markedly dysplastic granulocytes and precursors. MGG × 100.

is generally poor. A group of 797 patients entered into MRC trials had a 10-year survival of 16% [49]. Progression may be less rapid in children with a low blast percentage, for example 20–30%, and in adults with prior MDS and relatively low blast counts [379]. Among childhood cases, presentation is at a significantly younger age than AML, NOS, the WBC is lower and the bone marrow blast percentage is lower [383].

Immunophenotype

There is expression of myeloid antigens such as CD13 and CD33 [379]. CD34, TdT, CD7 and CD56 are often expressed. Expression of CD14 on blast cells has been found to be prognostically adverse [384].

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 [384].

Complex	Unbalanced*	Balanced
Three or more unrelated abnormalities (none of which would qualify a case to be categorized as AML with recurrent genetic abnormality)	del(5q) or unbalanced t(5q) −7 or del(7q) del(11q) i(17q) or t(17p) del(12p) or t(12p) −13 or del(13q) idic(X)(q13)	t(1;3)(p36.3;q21.2) t(2;11)(p21;q23.3)† t(3;5)(q25.3;q35.1) t(3;21)(q26.2;q22.1)† t(5;7)(q32;q11.2) t(5;10)(q32;q21) t(5;12)(q32;p13.2) t(5;17)(q32;p13.2) t(11;16)(q23.3;p13.2)†

AML, acute myeloid leukaemia; t-AML, therapy-related AML.
* In the 2016 revision of the WHO classification the del(9q) and monosomy 5 are no longer criteria
† As long as t-AML is excluded.
Adapted from Arber *et al.* 2017 [384].

Cytogenetic and molecular genetic features

By definition, the recurrent cytogenetic/genetic abnormalities that define definitive WHO categories of AML are absent. Cytogenetic abnormalities are often complex and unbalanced and may include abnormalities of chromosomes 5 and 7. Tetraploidy and near-tetraploidy are over-represented in this category of AML [385]. The cytogenetic abnormalities that qualify a case for inclusion in this WHO category are shown in Table 3.5 [384].

Characteristic molecular genetic abnormalities are mutation in *ASXL1* (35% of cases), *RUNX1* (17%), *IDH2* (20%) and *TET2* (15%) [386]. Somewhat less common are mutations in *U2AF1* (11.5%), *SRSF2* (8.5%) or *SF3B1* (4%) [387]. In one series mutations in *FLT3* and *DNMT3A* were uncommon [386]. In a series of childhood cases, *FLT3*-ITD was found in 10.8% and was associated with a worse survival [383].

Therapy-related myeloid neoplasms

Two broad groups of t-AML were recognized in the 2001 WHO classification [388]. In the first type, MDS and AML occurred following exposure to either alkylating agents (e.g. chlorambucil, busulfan, 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 [388]. The leukaemia could be of any FAB type but was rarely M3, and M6 was over-represented [388]. 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 [22,23,25,109,233,316,389–392].

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

Table 3.6 Cytogenetic abnormalities associated with therapy-related acute leukaemia [22–25,109,170,233,316,389–392].

Following alkylating agents and nitrosoureas	Following topoisomerase II-interactive drugs
Drugs incriminated	
Chlorambucil, busulfan, 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 3p−, 11q−, 12p−, −17, 17p− or other loss of 17p, 13q−,−18, 20q−, −21, 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)(q21.3q26.2) t(3;3)(q21.3;q26.2) t(6;9)(p23;q34.1) t(8;16)(p11.2;p13.3)	Chromosomal rearrangements with an 11q23.3 breakpoint (*KMT2A* often shown to be rearranged): t(1;11)(p32;q23.3)[†] t(1;11)(q21;q23.3) t(3;11)(p21;q23.3) t(3;11)(q25;q23.3) t(3;11)(q28;q23.3) t(4;11)(q21.3;q23.3)[‡] t(5;11)(q35;q23.3)[†] t(6;11)(q27;q23.3) t(9;11)(p21.3;q23.3) t(10;11)(p11;q23.3) t(10;11)(p13;q23.3) inv(11)(p14q23.3) t(11;11)(p13–15;q23.3) t(11;16)(q23.3;p13.3)[‡] t(11;17)(q23.3;p13) cryptic t(11;17)(q23.3;q25) t(11;19)(q23.3;p13.3)[‡] t(11;19)(q23.3;p13.1) t(11;21)(q23.3;q22) Chromosomal rearrangements with a 21q22.1 breakpoint (*RUNX1* often shown to be rearranged): t(1;21)(p36;q22.1) t(3;21)(q26.2;q22.1) t(7;21)(q31;q22.1) t(8;21)(q22;q22.1) t(16;21)(q24;q22.1) 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) [316] t(10;11)(q22~23;p15) [316] inv(11)(p15q22 or q23) t(11;17)(p15;q21) t(11;20)(p15;q11) Other: t(6;9)(p23;q34.1) t(8;16)(p11.2;p13.3) t(9;22)(q34.1;q11.2)[§] [316] t(15;17)(q24.1;q21.2) [109] inv(16)(p13.1q22) [109] t(16;16)(p13.1;q22) [109]

[*] Apparent monosomy 5 generally results from an unbalanced translocation with 5p sequence being retained.
[†] Therapy-related acute lymphoblastic leukaemia (ALL) [390].
[‡] Including therapy-related ALL [390].
[§] Therapy-related acute myeloid leukaemia, ALL or chronic myeloid leukaemia.

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, for example those with 11q23.3 breakpoints, quite a large proportion of cases are t-AML whereas in others, such as t(8;21)(q22;q22.1) (Fig. 3.56) and t(15;17)(q24.1;q21.2), 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 275) or mixed phenotype rather than myeloid; these cases are related to translocations with an 11q23.3 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 generally worse than that of *de novo* AML with the same cytogenetic abnormality. The uncommon cases of secondary leukaemia with t(8;21) or t(15;17) 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 [25,109]. In one series of nine patients with therapy-related acute promyelocytic leukaemia, prognosis was considered similar to that of *de novo* disease [393]. Cases involving the *KMT2A* gene certainly have a poor long-term prognosis despite an initially high complete remission rate [170,233]. In addition to the difference in cytogenetic abnormalities between disease induced by different types of agent, there are also differences in other molecular abnormalities [394]. For example, *TP53* and *RAS* mutations and *RUNX1* and *KMT2A* duplications and amplifications are particularly associated with alkylating agent-induced disease [394].

It is suspected that antimetabolites, such as azathioprine and fludarabine, can also cause t-AML [395]. The cytogenetic abnormalities described have been those characteristic of alkylating agent-related t-AML/MDS.

Acute myeloid leukaemia following radiotherapy or other radiation exposure is classified as t-AML. Some cases follow radio-iodine therapy [396].

In the 2008 WHO classification and its 2016 revision, 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 [397,398].

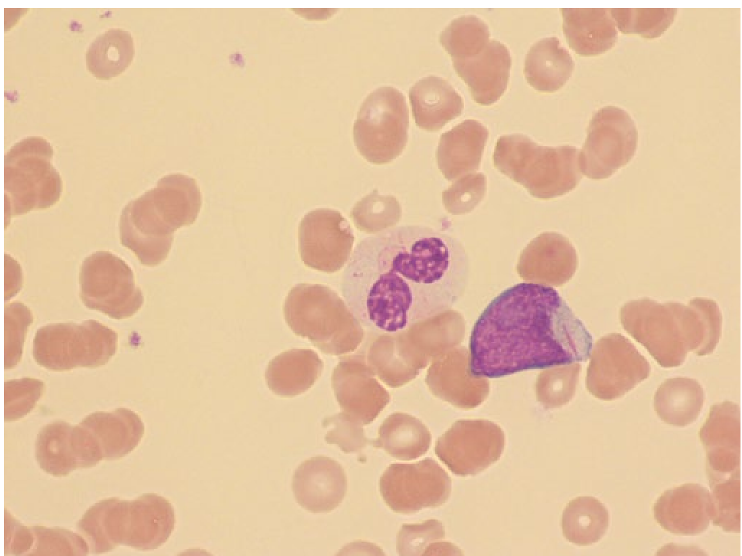


Fig. 3.56 PB film of a patient with therapy-related AML associated with t(8;21)(q22;q22.1) showing the usual features associated with this cytogenetic abnormality – a dysplastic neutrophil and a blast cell with a single long thin Auer rod. MGG × 100.

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 AML, NOS [399] 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.

The morphological subtypes of AML, NOS have been found not to be of prognostic significance [400] with the possible exception of pure erythroid leukaemia [399].

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, such as AML with t(8;16)(p11.2;p13.3), are discussed below. Some have cryptic chromosomal rearrangements. There are also recurrent molecular genetic abnormalities, for example *FLT3*-ITD.

Acute myeloid leukaemia with minimal differentiation

Acute myeloid leukaemia with minimal differentiation resembles the FAB M0 category (see page 14) 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.

Immunophenotyping usually shows expression of CD34, CD38, HLA-DR, sometimes CD13 and CD117, and somewhat less often CD33 [309]. CD7 and TdT may be expressed. Markers of maturation are negative. Although MPO is negative by cytochemistry, expression may be detected by flow cytometry [309].

Acute myeloid leukaemia without maturation

Acute myeloid leukaemia without maturation resembles the FAB M1 category (see page 18) 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.

The immunophenotype is similar to that of AML with minimal differentiation except that MPO is positive.

Acute myeloid leukaemia with maturation

Acute myeloid leukaemia with maturation (more correctly with granulocytic maturation) resembles the FAB M2 category (see page 21) except that cases meeting the criteria for the specific entities defined above, for example cases with t(8;21)(q22;q22.1), 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.

Immunophenotyping usually shows expression of MPO, CD13, CD33 and often CD34, HLA-DR and CD117. Markers of granulocytic maturation (CD11b, CD15 and CD65) are positive. CD7 may be expressed.

Acute myelomonocytic leukaemia

Acute myelomonocytic leukaemia resembles the FAB M4 category (see page 30) except that cases meeting the criteria for the specific entities defined above, for example cases with inv(16)/t(16;16) or with t(9;11) or other rearrangement of *KMT2A*, are excluded. A blast count (including promonocytes) of 20% in either the blood or bone marrow is sufficient for the diagnosis. The peripheral blood film may resemble that of chronic myelomonocytic leukaemia so that examination of the bone marrow is critical.

The immunophenotype resembles that of AML with maturation with the addition of expression of monocytic markers such as CD4, CD14 and CD64.

Acute monoblastic/monocytic leukaemia

Acute monoblastic/monocytic leukaemia resembles the FAB M5 category (see page 35) except that cases meeting the criteria for the specific entities defined above, for example cases with t(9;11) or other rearrangement of *KMT2A*, 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).

Immunophenotyping shows expression of HLA-DR, CD13, CD33, CD15 and CD65 [309]. CD34 and MPO are usually negative in monoblasts. More mature cells show expression of monocytic markers such as CD11c, CD14, CD64 and CD68, and CD7 and CD56 expression is not infrequent [309].

Acute erythroid leukaemia

The 2008 WHO classification recognized erythroleukaemia (erythroid/myeloid leukaemia), which was similar to the FAB category of M6 AML; in the 2016 revision, such cases are classified according to the number of blast cells as a percentage of all nucleated cells, and are not separately recognized. Pure erythroid leukaemia is defined by more than 80% of bone marrow cells being erythroid with 30% or more of erythroid cells being proerythroblasts; there is no significant myeloblastic component [399]. This rare condition was recognized in 1923 by Giovanni Di Guglielmo, who suggested the name ‘acute erythremic myelosis’ [401]. The designation ‘M6 variant AML’ was also previously used [402]. 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. There may be circulating erythroblasts. In histological sections there may be intrasinusoidal leukaemic cells. Cases with dysplasia of other lineages are assigned to this category rather than to ‘AML with myelodysplasia-related changes’ when the lack of a minimum of 20% blast cells precludes the latter diagnosis. Antigens that may be expressed include glycophorin A (CD235a) 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. CD71, the transferrin receptor, may be expressed but is similarly not lineage specific. CD34 and HLA-DR are often negative but CD117 may be positive. Cadherin-E is lineage specific and is useful for recognition of cases that do not express CD235a [403]. Complex karyotypes may be common [403] but data on cases that meet the 2016 WHO criteria are lacking. Prognosis is poor.

Some cases that would otherwise meet the WHO criteria for pure erythroid leukaemia are therapy-related and are therefore assigned to that category.

Acute megakaryoblastic leukaemia

Acute megakaryoblastic leukaemia resembles the FAB M7 category (see page 44) except that it is more precisely defined (blasts are at least 20% in the peripheral blood or the bone marrow and at least 50% of blasts are megakaryoblasts) and cases meeting the criteria for the specific entities defined above, for example acute megakaryoblastic leukaemia associated with t(1;22) and AML with myelodysplasia-related changes, are excluded. Babies and infants with myeloid neoplasms related to Down syndrome (see below) are also excluded. Some but not all cases have prominent fibrosis. There is an association with mediastinal non-seminoma germ cell tumours [404].

The blast cells may have cytoplasmic blebs, suggesting their lineage, or may appear undifferentiated. Differentiation to dysplastic megakaryocytes, including micromegakaryocytes, can occur. Cytopenia including thrombocytopenia is common, but some cases have thrombocytosis [399]. There may be giant or agranular platelets [399]. Immunophenotyping shows expression of CD41 and CD61 (and less often CD42b) in addition to CD13, CD33 and CD36. CD34, CD45 and HLA-DR are often negative [399].

Complex chromosomal abnormalities are common among non-Down syndrome children with acute megakaryoblastic leukaemia and lead to categorization as AML with myelodysplasia-related changes. The most frequent genetic abnormality is a cryptic inversion, inv(16)(p13.3q24.3) leading to a *CBFA2T3-GLIS2* fusion gene, and associated with a worse prognosis [405]. Other cases are associated with *NUP98-KDM5A*, while single cases have been reported with a number of other fusion genes [405]. Cases associated with *KMT2A* rearrangement [405] are assigned to that genetic category. In paediatric cases, poor outcome is associated not only with *CBFA2T3-GLIS2* (found in 16% of cases) but also with *NUP98-KDM5A* (9% of cases) and monosomy 7 (6% of cases) [406].

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. Clinicopathological features may include those resulting from histamine excess, hepatomegaly, splenomegaly, cutaneous involvement and lytic lesions [399]. Blasts of basophil lineage may be packed with basophilic granules (somewhat resembling acute promyelocytic leukaemia) (Fig. 3.57) or may have more sparse granules and cytoplasmic vacuolation (Fig. 3.58). They may have blocks or lakes of PAS-positive material. Basophil (or mast cell) differentiation can be confirmed by metachromatic staining with toluidine blue (Fig. 3.59). In contrast to mast cells, CAE may be negative. Only about 20% of cases show MPO positivity [309].

Immunophenotyping may show expression of CD11b, CD123, CD203c (also expressed on mast cells) and often CD9 and CD25 in addition to CD11b, CD13, CD33 and CD133 [40,309,399]. In contrast to mast cells, CD117 is negative [399]. Normal basophils are HLA-DR-negative but leukaemic basophils may be HLA-DR positive [309]. CD22 may be expressed [399].

The presence of t(9;22) and *BCR-ABL1* must be excluded. Cytogenetic abnormalities that have been observed include t(3;6)(q21;p21) [298] and t(X;6)(p11.2;q23.3) with *MYB-GATA1* [407].

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 [408]. Cases that meet the criteria for AML with myelodysplasia-related changes are excluded from this category. Patients may present with fever or bone pain [399]. Constitutional symptoms, fever and bone pain are typical [399]. Patients usually have pancytopenia

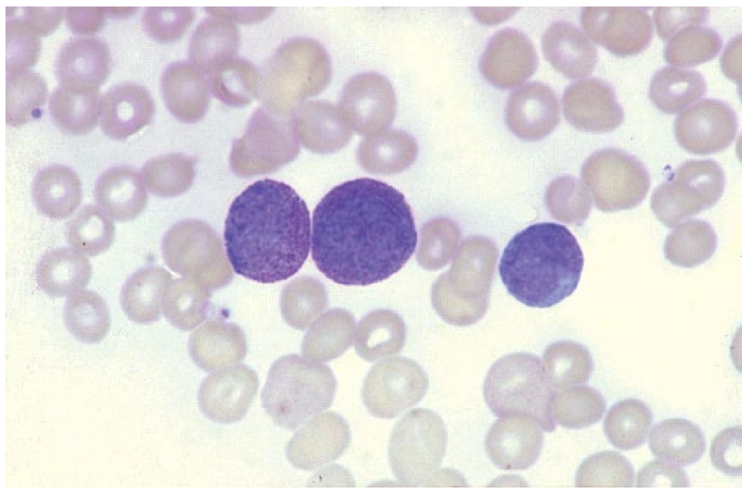


Fig. 3.57 PB film in acute basophilic leukaemia showing heavily granulated blast cells. MGG × 100.

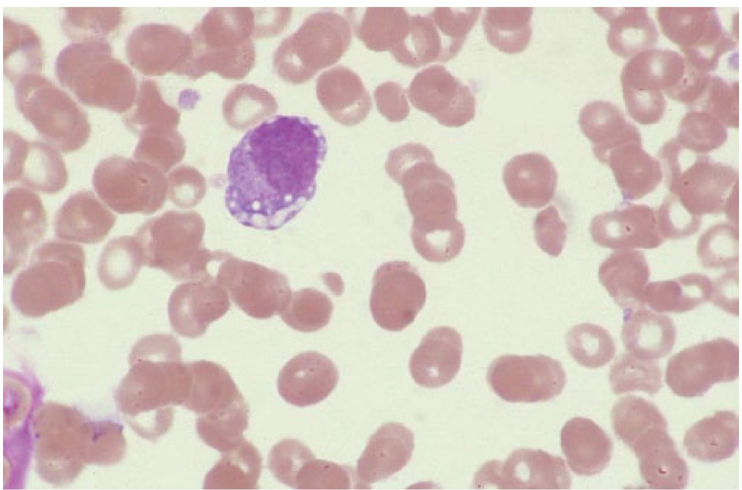


Fig. 3.58 PB film in acute basophilic leukaemia showing a vacuolated blast cell with scanty granules. MGG × 100.

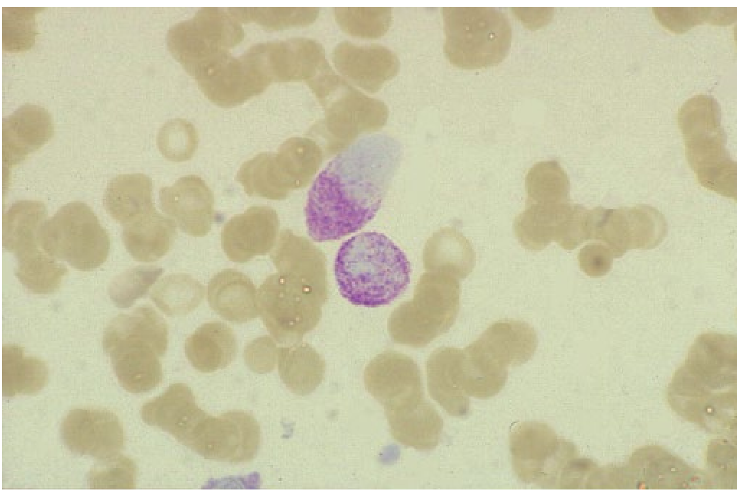


Fig. 3.59 PB film in acute basophilic leukaemia showing blast cells that stain metachromatically with toluidine blue. Toluidine blue × 100.

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. 3.60). The blast count is usually relatively low, for example 20–25% [399]. Blast cells are a variable mixture of myeloblasts or monoblasts, proerythroblasts and megakaryoblasts (Fig. 3.61). There may be prominent inflammatory changes including lymphoid nodules, increased plasma cells and increased vascularity [409]. Prognosis is poor. 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 [410].

Myeloid sarcoma

Myeloid sarcomas are solid extramedullary tumours that can be composed predominantly of cells showing granulocytic differentiation (granulocytic sarcoma) or predominantly of cells showing monocytic differentiation (monocytic sarcoma). Sometimes eosinophils are prominent. Common sites of involvement include skin and soft tissues, lymph nodes, the gastrointestinal tract, bone and testis; multiple sites may be involved. 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)(q22;q22.1) (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 (TAM) and acute megakaryoblastic leukaemia [411]. 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.

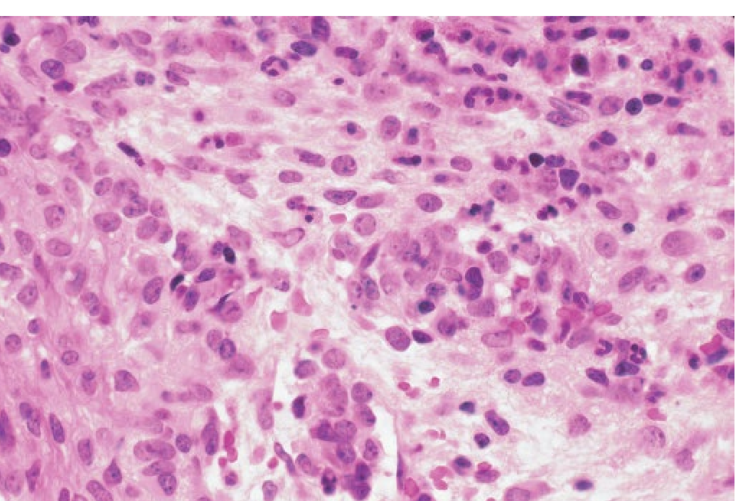
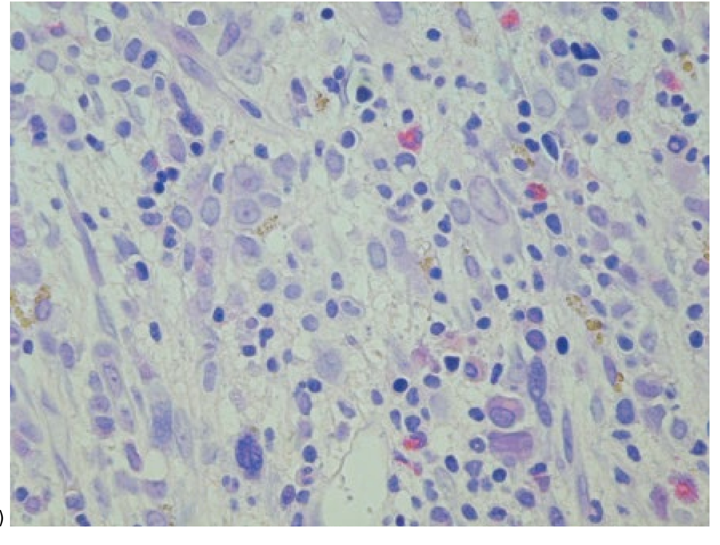
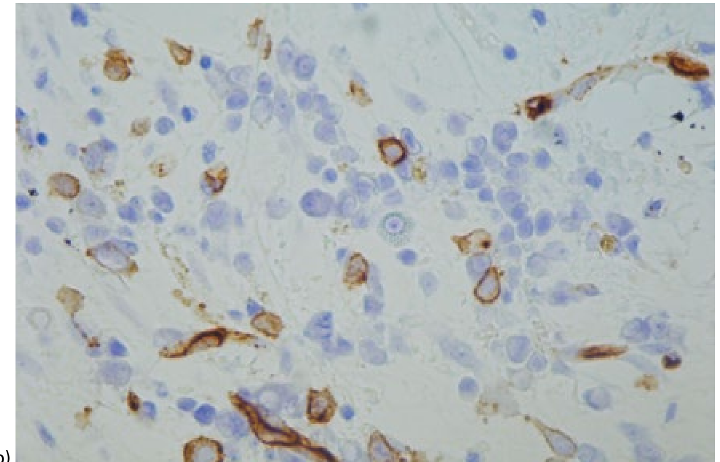


Fig. 3.60 Trephine biopsy section from a patient with acute panmyelosis. H&E × 100. (With thanks to Dr Richard D. Brunning.)


(a)


(b)

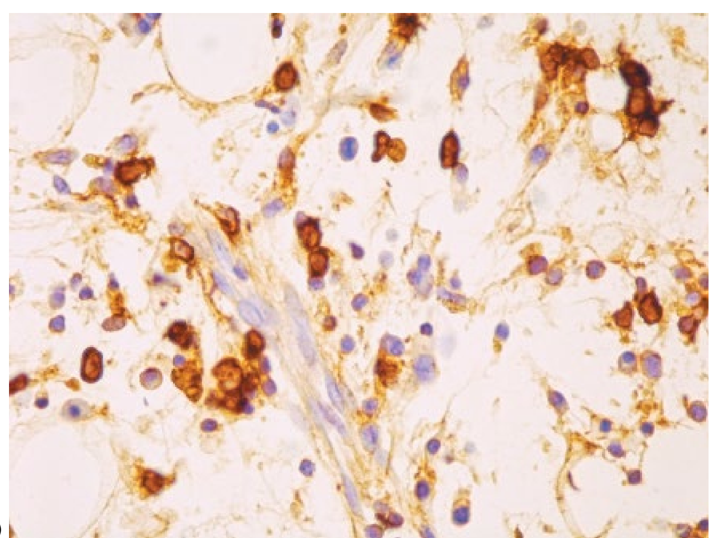

(c)

Fig. 3.61 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. (c) Myeloblasts are highlighted by their lysozyme content. Immunoperoxidase × 60.

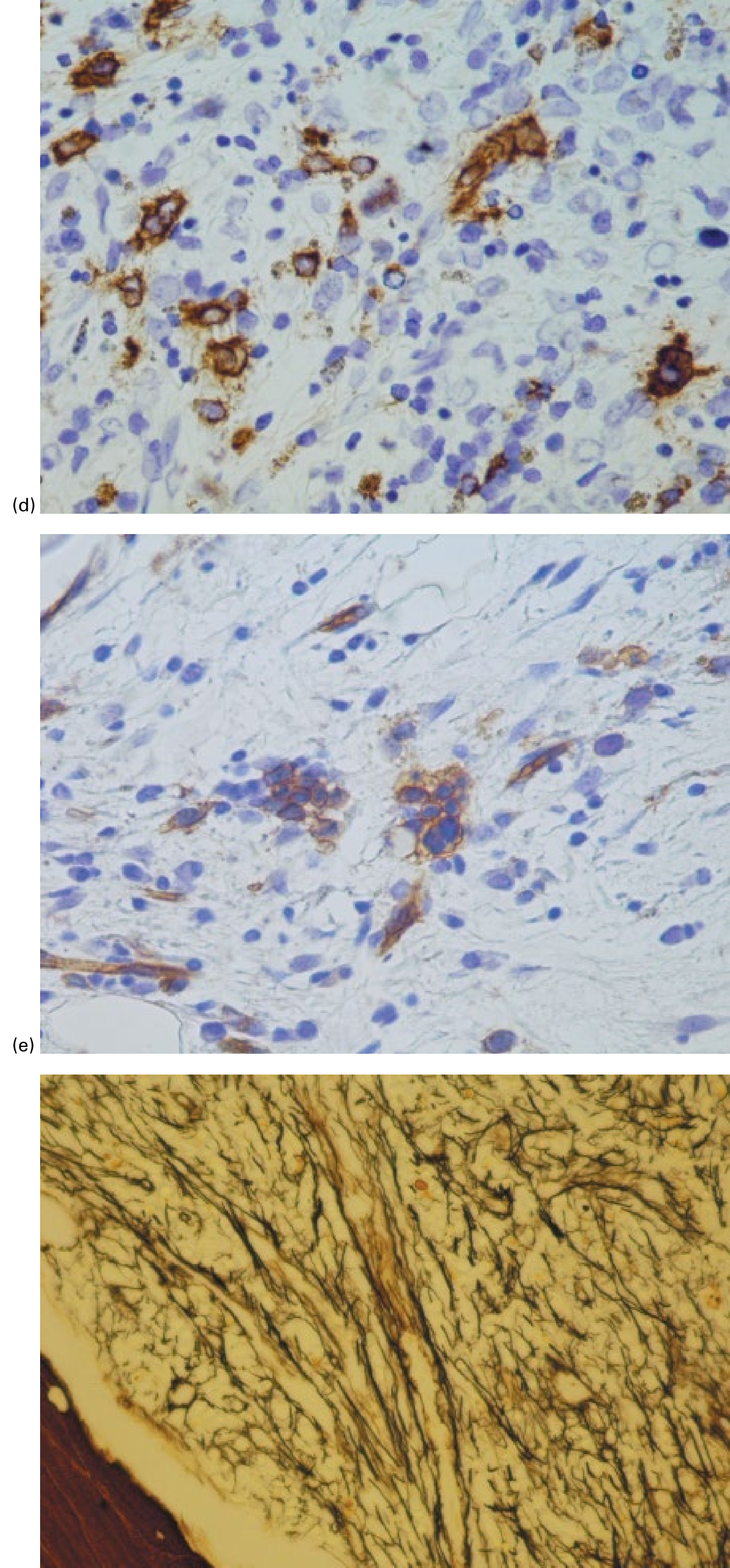


Fig. 3.61 (*Continued*) (d) Megakaryoblasts and dysplastic megakaryocytes are highlighted by detection of CD61. Immunoperoxidase × 60. (e) Clusters of erythroblasts are highlighted by binding to a lectin derived from *Ulex europaeus*. Immunoperoxidase × 60. (f) Heavy reticulin deposition, grade 4 of 4 since collagen was also present. Reticulin stain × 60.

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 was previously sometimes regarded as a leukaemoid reaction. However, in a number of cases there has been an additional clonal cytogenetic abnormality in the proliferating cells and, by molecular analysis, clonality can be shown in all [412–415]. It is now clear that TAM is actually a spontaneously remitting leukaemia with mutation of the *GATA1* gene being consistently present [416]. The designation transient leukaemia of Down syndrome may therefore be preferred. 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 [417]. Using Sanger sequencing/denaturing high-performance liquid chromatography (DHPLC), *GATA1* mutations were found in 8.5% of 200 babies with Down syndrome, all of whom had more than 10% circulating blast cells and, using next-generation sequencing, low-abundance mutant clones were found in 20% of 88 babies who were negative on Sanger sequencing/DHPLC; there were no distinguishing features in the latter group, who could be regarded as having 'silent TAM' [418]. TAM may arise in a fetal liver haemopoietic stem or progenitor cell [419].

Babies with TAM often have hepatosplenomegaly, jaundice and anaemia; less often they have liver failure or hydrops fetalis [420] with pericardial and pleural effusions and ascites. There may be liver fibrosis. Renal dysfunction, a rash and a bleeding diathesis are seen in a minority of cases [419]. Some affected fetuses die *in utero*, and some babies die as a result of bone marrow dysfunction, liver failure, DIC or renal failure. A higher WBC, premature birth, abnormal liver function tests, hepatomegaly, increased conjugated bilirubin, ascites, haemorrhagic manifestations and, not surprisingly, failure to enter spontaneous remission all correlate with early death [415,421–423]. In one study 23% of babies with TAM died in the first 6 months of life [422]; in a second series, the death rate was 15% [421]; and in a third large study the death rate was 21%, with TAM-related deaths being 10% [423]. Phenotypically normal neonates with mosaic trisomy 21 can also develop TAM, as can babies with mosaic Down syndrome and in babies without Down syndrome who have both trisomy 21 and a *GATA1* mutation confined to their haemopoietic cells [424]. Babies with TAM and silent TAM are at risk of subsequent acute megakaryoblastic leukaemia, 3/17 and 1/88, respectively [418]. Babies with mosaic trisomy 21 and with trisomy 21 confined to haemopoietic cells are also at risk [424].

Typically there is an increase in the WBC with neutrophilia and the presence of myelocytes and blast cells but anaemia is uncommon [419]; basophilia is common and sometimes there is eosinophilia. The basophil count is occasionally greatly elevated [425]. There may be 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 [414]. There may be giant platelets, hypogranular 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 3.62–3.64). The abnormal cells are often megakaryoblasts but sometimes have features of primitive erythroid cells or of basophiloblasts [426].

The differential diagnosis of TAM includes not only other forms of congenital leukaemia but also the haematological features of Down syndrome itself. If peripheral blood blast cells are more than 20%, TAM is probable, but lower numbers can be seen in Down syndrome babies without TAM, and thrombocytopenia is also common [419].

The immunophenotype in TAM is characteristic [421,427]. Blasts are positive for CD7, CD13, CD33, CD34, CD36, CD38, CD71 and

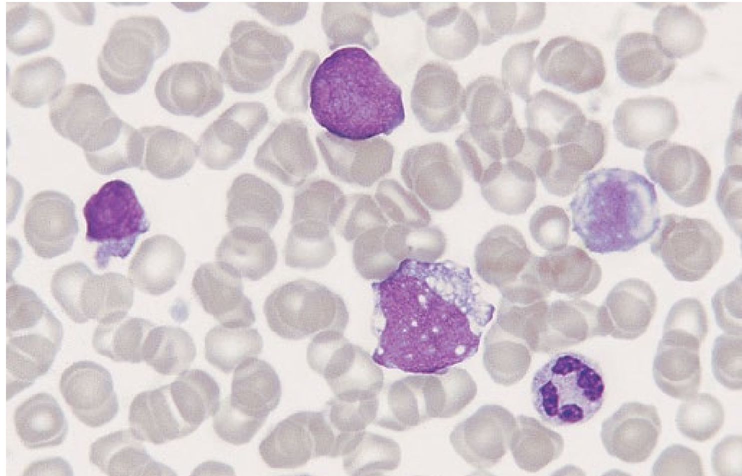


Fig. 3.62 PB of a neonate with transient abnormal myelopoiesis (TAM) of Down syndrome showing a neutrophil, a giant platelet, an unidentifiable abnormal cell, a blast cell and a micromegakaryocyte. The blast cells were demonstrated to be megakaryoblasts by immunophenotyping. MGG × 100.

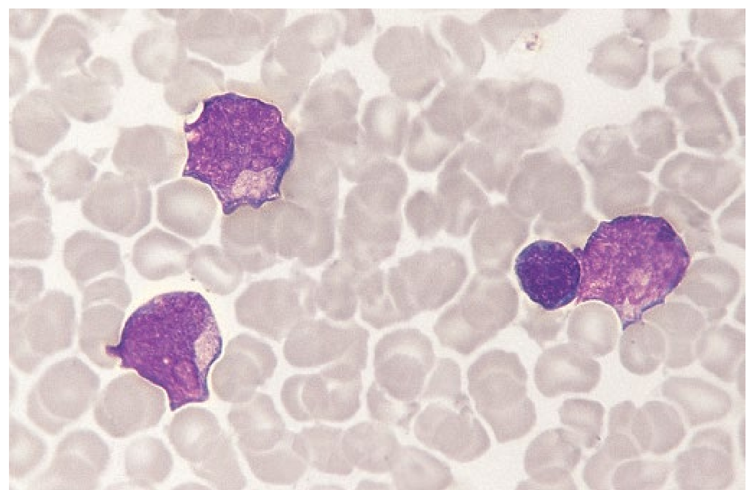


Fig. 3.63 BM film of the same patient as shown in Fig. 3.62 showing a lymphocyte and three pleomorphic blasts. MGG × 100.

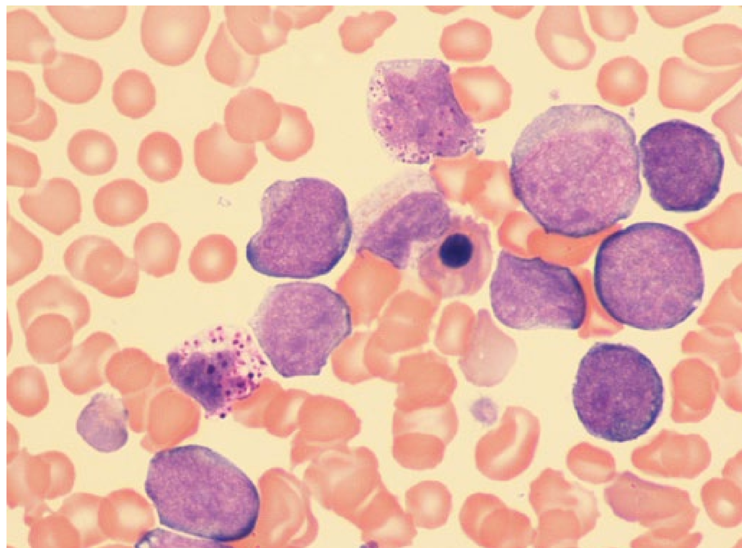


Fig. 3.64 PB film from another neonate with TAM in whom differentiation was to myeloblasts, megakaryoblasts, micromegakaryocytes, erythroblasts and basophils. MGG × 100.

CD117. CD41, CD42b and CD61 are usually expressed and CD56 is expressed in about 80% of cases. HLA-DR is positive in about a third of cases [309]. There may also be expression of glycophorin A (CD235a). In one study blast cells expressed receptors for erythropoietin, thrombopoietin and interleukin 3 [421] but in another erythropoietin receptor was not detected [427].

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 GATA in promoting megakaryocyte maturation. It is likely that the leukaemogenic mutation in TAM and acute megakaryoblastic leukaemia involves interaction of a mutated *GATA1* gene and one or more chromosome 21 genes that are present in the genome as three rather than two copies. One such gene that may be implicated is *ERG* at 21q22, expression of which favours megakaryocyte differentiation [428].

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. In one study the presence of clonal cytogenetic abnormalities during TAM correlated with future development of leukaemia [415], but in a second larger study it was not predictive of future leukaemia or of prognostic significance [421].

A prospective study of 146 babies with TAM found an overall 5-year survival of 85% and an event-free survival of 63% [421]. In those with adverse prognostic features (high WBC, ascites, bleeding diathesis and preterm birth), low-dose cytarabine therapy was demonstrated to be of benefit [421]. 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%) [421]. In another large study, transformation occurred in 16% of infants at a median of 441 days (range 118–1085) [423].

Myeloid leukaemia associated with Down syndrome

Six percent of UK children with AML have Down syndrome [428]. In a fifth to a quarter of babies who have shown evidence of TAM, acute myeloid leukaemia subsequently develops [414,415,421], 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 [429]. As would be expected, not all babies with a *GATA1* mutation at birth developed AML [429]. All cases of Down syndrome-related AML show a *GATA1* mutation [430] and when there has been preceding TAM the mutation is the same [416,421]. Multiple independent *GATA1* mutations may be present [429].

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 [428]. In 14 UK children the median age of onset was 2.2 years [432]. In the WHO classification, MDS and AML are grouped together as ‘myeloid leukaemia associated with Down syndrome’ [411]. The acute leukaemia of Down syndrome is usually FAB type M7 (Fig. 3.65) and less often M6 [415,433]. Bone marrow reticulin is increased [411]. Cases of FAB M1 and M2 have been reported but sometimes the diagnosis has been revised to M6 or M7 on review [415,428,431]. 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 [431].

Immunophenotyping usually shows expression of platelet antigens such as CD41, CD42 and CD61 [431,434,435]. CD36 is often expressed. Myeloid antigens such as CD13 and

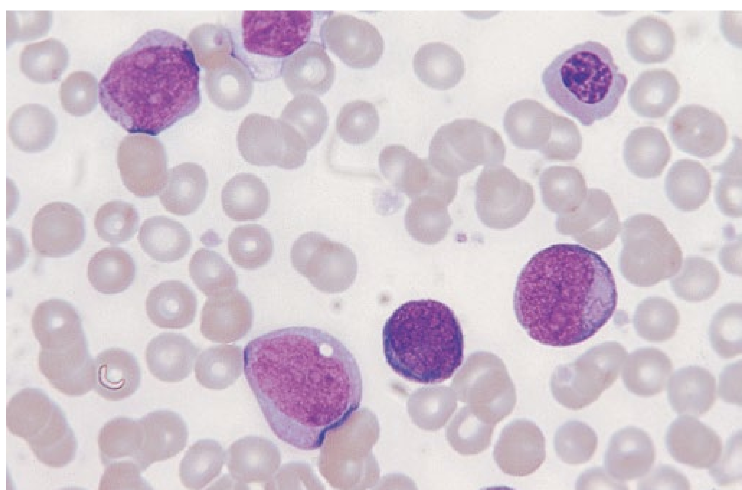


Fig. 3.65 PB film from a patient with Down syndrome with FAB M7 AML; blasts are pleomorphic with no specific distinguishing features. The nature of the leukaemia was demonstrated by showing expression of CD61. MGG × 100.

CD33 are expressed in approaching 80% of cases, and coexpression of CD7 and CD117 is even more common [427,431]. Glycophorin (CD235a) is expressed in about half of patients [431]. CD34 is expressed in about half (in comparison with about 90% in TAM), and CD56 may be expressed [427]. 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 [415]. Those initially showing only the constitutional abnormality often also show an acquired abnormality at the AML stage of the disease. Trisomy 8 is common. Cytogenetic abnormalities, found in three-quarters of children with Down syndrome and AML, differ from those in non-Down syndrome childhood AML [436]. The common balanced translocations are uncommon in Down syndrome and instead unbalanced abnormalities are common: −5, −7, dup(1q), del(5q), del(6q), del(7p), dup(7q), +8, +11, del(16q) and acquired +21 [436,437].

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 [438]. Recurrently mutated genes have been identified that are likely to be implicated in progression to AML including *JAK1*, *JAK2*, *JAK3*, *MPL*, *KRAS*, *MRAS*, *SH2B3* (loss-of-function mutation), *STAG2*, *RAD21*, *SMC3*, *SMC1A*, *NIPBL* and *CTCF* [405]. There may also be mutations in genes encoding epigenetic regulators, such as *PRC2* and *SUZ12* [405].

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 [88,439]. The leukaemia is very susceptible to cytarabine, anthracyclines, mitoxantrone and etoposide [433]. Complete remission rate is around 90%, and 10-year survival and event-free survival is nearly 80% [431].

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 [440], and in the 2008 WHO classification and its 2016 revision it has been designated blastic plasmacytoid dendritic cell neoplasm [441]. There is an association with AML, MDS and chronic myelomonocytic leukaemia [441,442] suggesting an origin from a

myeloid stem cell. However, this blastic condition must be distinguished from mature plasmacytoid dendritic cell proliferation in association with other myeloid neoplasms, a condition that is also likely to be clonal and neoplastic.

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). 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

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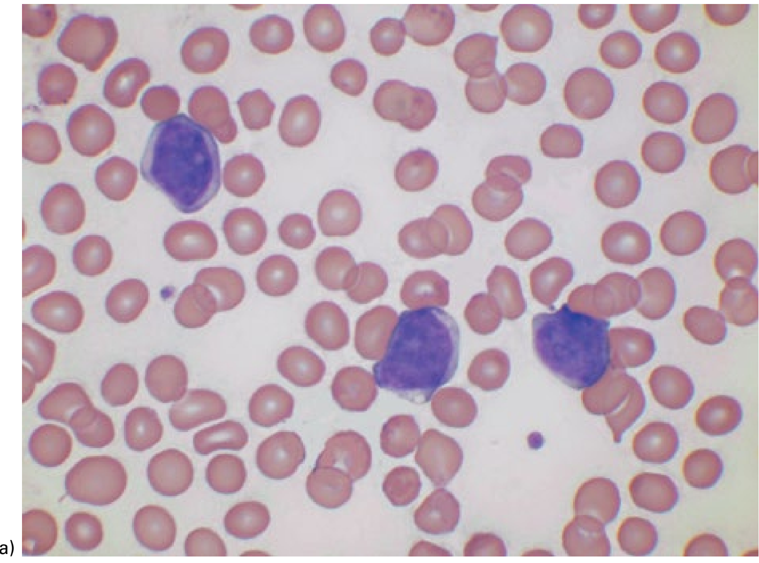


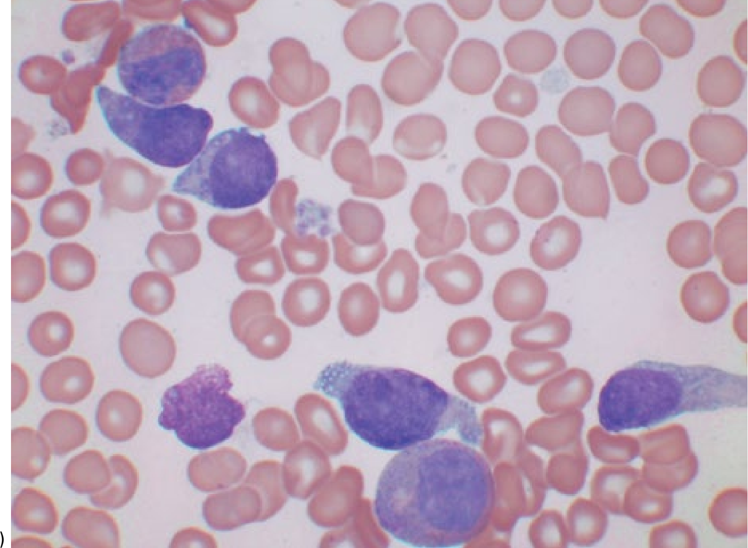
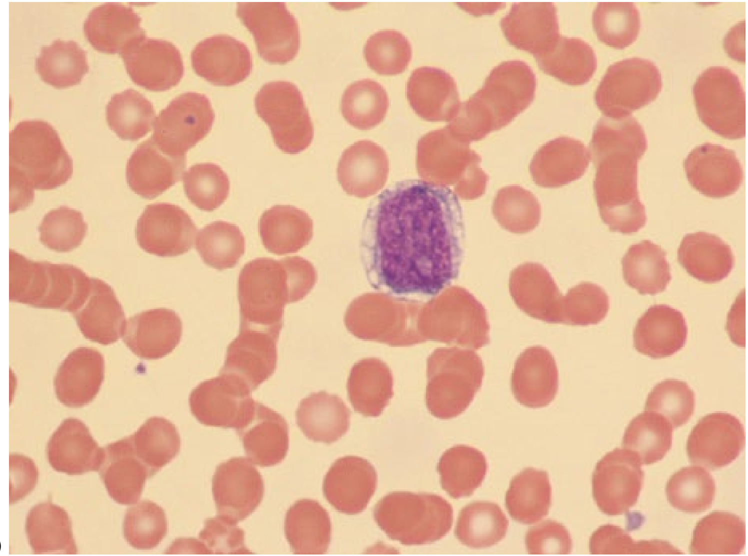
(b)


Fig. 3.66 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.

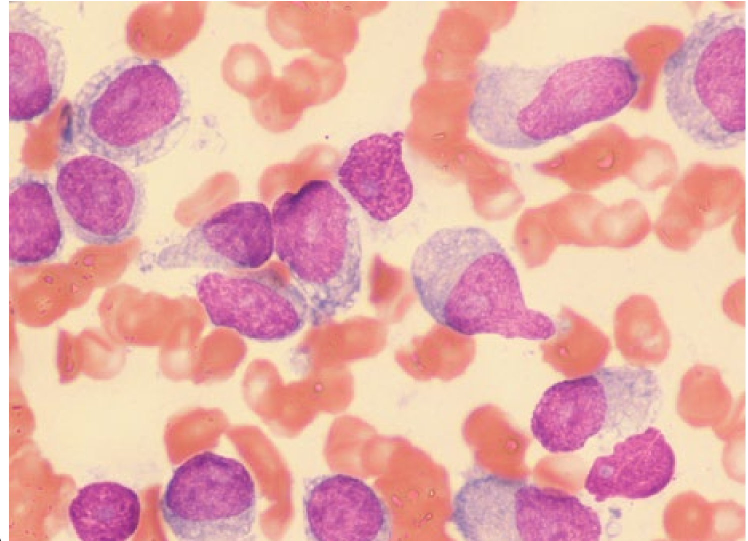
bone marrow is usually involved and blood involvement is often present (Figs 3.66 and 3.67). Cytopenias are common. Cells are either small to medium or medium to large in individual patients, with regular or irregular nuclei and a variable amount of vacuolated, agranular cytoplasm with an irregular surface membrane. Prominent cytoplasmic tails are often present and there may be small vacuoles. There are coexisting myelodysplastic features in about a third of patients [443]. Lack of alpha-naphthyl butyrate esterase activity can help in making a distinction from acute monoblastic leukaemia.

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 (myeloperoxidase



(a)



(b)

Fig. 3.67 Blastic plasmacytoid dendritic cell neoplasm. (a) PB film showing a neoplastic cell with heavy cytoplasmic vacuolation. (b) BM film showing blast cells with cytoplasmic tails and cytoplasmic vacuoles. (With thanks to Dr Wenchee Siow.)

and lysozyme negative, CD117 only occasionally positive) [441,444]. The only T-lineage antigens expressed are CD4, often CD7 and sometimes CD2. 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 [445]. CD45 expression is usually low [445]. There is often also expression of CD36, CD38, CD68 (about half of cases), CD71 and strong HLA-DR [440,445]. CD43 and plasmacytoid dendritic cell-associated markers, CD68, CD303 (BDCA-2), CD304 (BDCA-4), TCL1A and CLA, as well as CD123, are expressed [446]. Some cases (up to a third) have shown expression of TdT [447]. CD13 and CD33 are often expressed but expression is weak [444]. CD68 may show dot-like cytoplasmic expression [441]. CD34 is usually negative. BCL2, BCL6, MUM1/IRF4 and S100, antigens not expressed by normal plasmacytoid dendritic cells, may be expressed [441].

Cytogenetic and molecular genetic features

Cytogenetic analysis shows clonal abnormalities, usually complex, in two-thirds of patients [448]. Rearrangements preferentially involve 5q, 17p, 12p, 13q, 6q, 15q with del(5q) being a frequent feature; chromosomes 9, 13 and 13q may be lost [440,443,448]. *TET2* mutations are common [443]. An association with t(11;19)(q23;p13) and *KMT2A* rearrangement [449] emphasizes the relationship of this leukaemia to AML. Haploinsufficiency for the tumour suppressor gene, *NR3C1*, is found in about a quarter of patients and is associated with a more adverse prognosis [450].

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); *KAT6A-CREBBP*

Acute myeloid leukaemia associated with t(8;16)(p11.2;p13.3) (Fig. 3.68) is a rare variant of AML, comprising fewer than 1% of cases [15]. Most cases occur *de novo* but there is a higher prevalence among cases of therapy-related AML [170,316,451]. 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 [452–454]. 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

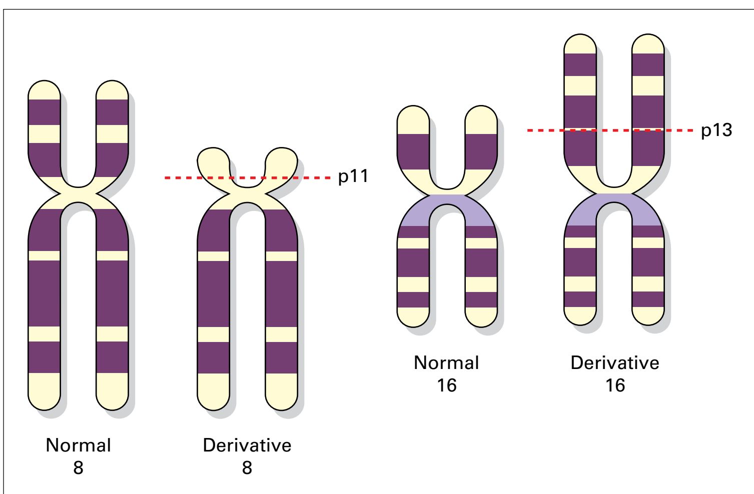
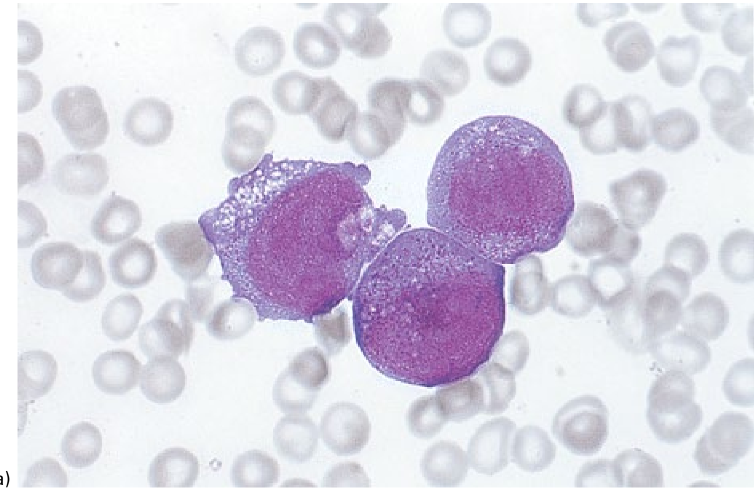


Fig. 3.68 Diagrammatic representation of t(8;16)(p11.2;p13.3) (modified from reference 2).


(a)

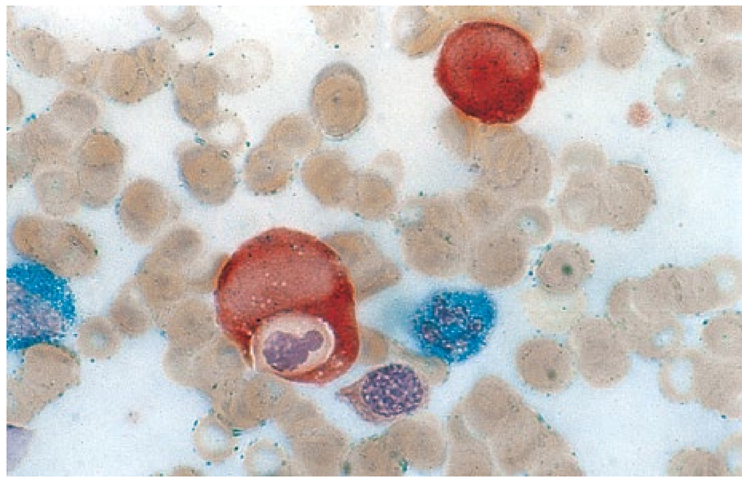

(b)

Fig. 3.69 BM aspirate from a patient with FAB M5 AML associated with t(8;16)(p11.2;p13.3). (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. (With thanks to Professor Daniel Catovsky, London.)

children, and if therapy-related cases are excluded the median age is low. Spontaneously remitting congenital leukaemia has occurred [269,455]; in one series all seven neonates with congenital acute leukaemia who were observed without treatment remitted, with three remaining in continuous remission [456]. A prominent haemostatic abnormality, sometimes interpreted as DIC and sometimes as increased fibrinolysis, is common. Extramedullary disease, including skin infiltration, is common [454]. The prognosis in adults is poor, with a median survival of 4.7 months in 13 patients [451], and a 5-year survival of 33% in another series of 13 patients, 9 of whom were adults [457]. However, in a series of 62 paediatric patients the 5-year overall survival was 59%, equivalent to a reference cohort of other paediatric cases [456].

Many cases described have shown haemophagocytosis by leukaemic cells (Fig. 3.69), 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. Usually blast cells are strongly positive for both myeloperoxidase and non-specific esterase [451].

Immunophenotype

The characteristic immunophenotype is positivity for HLA-DR, CD13 (two-thirds of cases), CD33, CD15, CD65 and myeloperoxidase [451,453,454].

Monocyte antigens, CD14, CD64 and CD11b are usually positive as are CD4 and CD56 [451]. CD34, CD117, CD133 and TdT are usually negative [451].

Cytogenetic and molecular genetic features

This translocation involves *CREBBP*. There is fusion of the *KAT6A* gene (previously known as *MOZ* then as *MYST3*) gene on chromosome 8 with the *CREBBP* gene (previously known as *CBP*) from 16p13 [458]. It appears to be the *KAT6A-CREBBP* fusion gene that is important in leukaemogenesis rather than the *CREBBP-KAT6A* gene [459]. Secondary chromosomal abnormalities include trisomy 1 and trisomy 8 [244]. The gene expression profile is similar to that of AML with *KMT2A* rearrangement but can be distinguished [451].

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) with *KAT6A-EP300*, t(3;8;17)(q27;p11;q12) [454] and t(1;16;18)(p13;p13;p11) [455], and also with an inversion of chromosome 8, inv(8)(p11q13), with *KAT6A-NCOA2* (previously *MOZ-TIF2*) [460]. One case of t-AML associated with t(8;22)(p11;q13) has been reported [461]. Haemophagocytosis by leukaemic blast cells has also been a feature of cases with t(8;22) and inv(8) [460,462].

Acute myeloid leukaemia with t(16;21)(p11.2;q22.2); *FUS-ERG*

t(16;21)(p11.2;q22.2) 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 [463,464]. 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 [48]. The *FUS-ERG* fusion gene can be detected by RT-PCR.

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 [206,404,465,466] but occasionally arising in an ovarian germ cell tumour [467]. 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 [468]. A variety of FAB types have been described. The commonest is M7 but M4, M5, M6, malignant histiocytosis and MDS (FAB refractory anaemia with excess of blasts 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; both refractory anaemia with ring sideroblasts [469] and refractory anaemia with excess of blasts [470], 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 [471].

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 [472–492]. Furthermore, trisomy 13 has been associated with mutation of *SRFS2* and *RUNX1*, often coexisting, and with a very poor prognosis [493].

Table 3.7 Some of the genetic categories of acute myeloid leukaemia not involving 11q23.3 or the *KMT2A* gene [270,279,297,472–492]; previous gene names are shown in parentheses.

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	[472]
t(1;11)(q23;p15)	FAB M2 (t-AML)	*NUP98-PRRX1* (*PMX1*)	[473]
t(1;12)(q21;p13)	A case of FAB M2 AML	*ETV6* (*TEL*)*-ARNT*	[474]
t(1;21)(p36;q22)	t-AML	*RUNX1* rearranged	[475]
t(2;11)(q31;p15)		*HOXD13-NUP98*	
t(3;5)(q25.1;q34)	AML or MDS	*NPM1-MLF1*	[297]
t(4;12)(q11-22; p13)	FAB M0 and M2 AML	*CHIC2* (*BTL*)*-ETV6*	[476]
Cryptic t(5;11)(q35;p15.5), often with normal cytogenetics or 5q−	Childhood and less often adult AML (FAB M1, M2, M4 or M5)	*NSD1-NUP98* and *NUP98-NSD1*	[477,478]
t(5;12)(q31;p13)	Case of AML with eosinophilia	*ACSL6* (*ACS2*)*-ETV6*	[479]
t(7;11)(p15;p15)	FAB M2 AML, in Chinese and Japanese; trilineage myelodysplasia	*NUP98-HOXA9* or *NUP98-HOXA13*	[279,480,481]
t(7;12)(p15;p13)		*ETV6* rearranged	
t(7;12)(q36;p13), often cryptic	*c.* 30% of infant AML (with poor outcome)	*MNX1* (*HLXB9*)*-ETV6* or *MNX1* overexpression	[482]
t(8;11)(p11.2;p15)	FAB M1 AML (one case)	*NUP98-WHSC1L1* (*NSD3*)	[483]
t(9;11)(p22;p15)	AML	*PSIP1* (*LEDGF*)*-NUP98*	[484]
t(9;12)(q34;p1?) (cryptic)	AML with trilineage dysplasia and clonal eosinophils	*ETV6-ABL1*	[485,486]
t(10;11)(p12.2; q14.2)	FAB M0, M1, M2, M4 and M5 AML, MPAL	*PICALM-MLLT10* (*AF10*), less often *MLLT10* (*AF10*)*-PICALM*	[270,487]
t(10;16)(q22;p13)	Childhood FAB M5a AML	*KAT6B-CREBBP* (*MORF-CBP* or *MYST4-CREBBP*)	[488]
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*	[489,490]
t(12;15)(p13;q25)	A case of FAB M2 AML	*ETV6-NTRK3* (*TRKC*)	[491]
t/dic(12;20)(p12–13;p11.2–q13)[†]	AML or MDS	Not known	[489]
t(12;22)(p13;q11)	Various FAB categories (M1, M4, M7)	*MN1-ETV6*	[492]
Normal	A case of FAB M0 AML	*SET-NUP214* (*CAN*)	

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

In addition, there are significant genetic groupings that are of biological and prognostic relevance that have not yet been incorporated into the WHO classification [353] (see below).

Other recurrent 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*, biallelic *CEBPA* and *RUNX1* mutations, the mutations have been recognized as defining 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 [329]. *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 prognostically unfavourable *FLT3*-ITD is associated with aberrant expression of CD7, this being observed in 11 of 15 patients with normal cytogenetic analysis and *FLT3*-ITD [494]. Overexpression of wild-type *FLT3* is found in 10–15% of patients with AML and is prognostically adverse [495]; both *FLT3*-ITD and *FLT3* overexpression are associated with FAB M4 and M5 subtypes and high expression of CD11b, CD14 and CD123 [495]. Cryptic translocations, some of considerable prognostic importance, are found within the group of patients with normal cytogenetics. The very adverse significance of *NUP98-NSD1*, found in 16% of paediatric cases and 2.5% of adult cases, has been attributed to an association with *FLT3*-ITD [478,496]. *DNMT3A* mutation is found in about 22% of patients, particularly those with a normal karyotype; there is overlap with *NPM1*, *IDH1*, *IDH2* and *FLT3* mutations but not with t(8;21), t(15;17), inv(16) or *KMT2A* rearrangement [497]. *DNMT3A* mutation has independent adverse prognostic significance [497]. An *IDH1* mutation was found in 6.6% of 1414 patients and correlated with female gender, FAB M1 morphology, intermediate risk cytogenetics (including trisomy 8 and normal cytogenetics), *NPM1* mutation, *KMT2A*-PTD and worse prognosis [498]. 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 [499].

Both *de novo* AML and t-AML can be associated with *KMT2A*-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 [500]. Prevalence rises with age and has varied from 3% to 6% in a large series of patients [501]. In patients with normal cytogenetics it is higher, ranging from 5% to 8% in large series [501]. The highest prevalence is in patients with trisomy 11, among whom about a third have *KMT2A*-PTD [501]. This genetic abnormality has been associated with a poor prognosis [225,501,502], but in a group of patients with *KMT2A*-PTD and normal cytogenetics who received intensive treatment the prognosis was not adverse [503]. *FLT3*-ITD may coexist [501].

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 [81,178,239,351,372,495–533].

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) in some studies but in one study only in those with t(8;21) [239]
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 [504]	15–40%; 36% in cytogenetically normal cases [499]	Adverse in multiple genetic subtypes including acute promyelocytic leukaemia and mutated *NPM1*; adverse with normal cytogenetics but possibly only in those with *NPM1* mutated [239,372,505]
FLT3-TKD (tyrosine kinase domain mutation)		9% of cytogenetically normal cases [499]	Data are conflicting [81,178,506]
FLT3 overexpression	Particularly myelomonocytic and monocytic/monoblastic categories	15%	Adverse [495]
KMT2A-PTD (partial tandem duplication)	Found in a third of patients with trisomy 11 and in 5–10% of cases with normal karyotype	3–6%; 5–8% of cytogenetically normal cases	Adverse [239,501,507] but not an independent risk factor
WT1 mutation		10% of cytogenetically normal cases	Adverse in association with normal karyotype [508], in some but not all studies only if associated with *FLT3*-ITD [509,510]
NRAS	t(8;21), inv(16), t(16;16) and normal cytogenetics	About 15%,	Uncertain, may predict response to cytarabine [511]
JAK2 V617F	Associated particularly with FAB types M1 and M2	4%	Not prognostic [512]
TET2 mutation	Associated preferentially with subtypes other than acute megakaryoblastic leukaemia; associated with older age and higher white cell count	12–17% of cytogenetically normal cases; 13% overall, associated with normal and intermediate-risk cytogenetics	Reduced OS, reduced survival in patients with intermediate-risk cytogenetics [513,514]
DNMT3A mutation	Associated preferentially with normal karyotype and with *NPM1* and *IDH1* mutations and *FLT3*-ITD; increases with age [511]	22% overall, 37% in those with a normal karyotype	Reduced DFS and OS, independently of age and *FLT3* mutation; may do better with higher doses of daunorubicin [351,497]
IDH1 mutation	Associated preferentially with female gender, intermediate risk or normal cytogenetics, *NPM1* mutation and *KMT2A*-PTD	6.6% of 1414 patients	Worse prognosis [498,511]

(Continued)

Table 3.8 (Continued)

Gene that is mutated or abnormally expressed	Associations	Frequency	Significance
IDH2 mutation	*IDH2*^{R140} associated with *NPM1* mutation and increases with age; *IDH2*^{R172} usually occurs alone; cytogenetics usually normal	10% of 1473 patients [515]	*IDH2*^{R140} is prognostically favourable; *IDH2*^{R172} is prognostically adverse [511,515]
ASXL1 mutation	Associated with wild-type *NPM1*, mutated *CEBPA*, absence of *FLT3*-ITD, mutation of *RUNX1*, *SRSF2* and *IDH2* and AML following MDS; increases with age [511]	10.6% of cytogenetically normal cases; 15.4% overall [507]	Prognostically adverse in those with normal karyotype [516] and overall [239,507]
TP53 mutation	Associated with complex karyotype, −5, 5q−, −7, 7q−; increases with age	11.5%	Highly adverse [507,511]
GATA2 mutation	Associated with biallelic *CEBPA* mutation	9.9% of intermediate risk AML	Prognostically good [517]
GATA2 overexpression		Expression higher than in normal marrow in 65% of childhood cases	Prognostically poor [518]
High *LEF1* expression		Half the cohort	Prognostically favourable in cases with normal cytogenetics [519]
PHF6 mutation			Prognostically adverse [239]
BAALC overexpression (defined as above the median value)	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 [499,505,520]
ERG overexpression		37% in cytogenetically normal cases [499]	Adverse in those with normal karyotype; adverse in children with *KMT2A*-rearranged AML [499,505,520– 522]
MN1 expressed [505]			Adverse in those with normal karyotype
MECOM (*EVI1*) overexpressed			Reduced OS and RFS in those with normal karyotype [521] but not an independent risk factor in childhood AML [523]; adverse in adult AML; prognosis may be improved with all-*trans*-retinoic acid [524]
Low *PRAME* expression			Reduced OS and RFS in those with normal karyotype [521]

Table 3.8 (Continued)

Gene that is mutated or abnormally expressed	Associations	Frequency	Significance
High *VEGFC* expression		Defined as top 50%	Adverse [525]
High *MLLT11* (*AFQ1*) expression			Worse prognosis in cytogenetically normal cases [526]
High *ARC* expression		Defined as top third	Upper third prognostically worse OS than middle and lower thirds [527]
CD25 expression	CD123 expression, *FLT3*-ITD, *DNMT3A* and *NPM1* mutations	13%	Prognostically adverse [528]
MicroRNA signature within genetic subtypes			Reduced event-free survival [529]
High *MIR3151* expression			Shorter DFS and OS in normal karyotype AML [530]
High *MIRN9* expression			Shorter RFS and OS [531]
High *LGALS3* expression	Older age, monocytic differentiation, CD4 expression and *PTPN11* mutation	Defined as top half	Lower CRR and shorter OS [532]
High *CRM1* expression	Higher WBC, higher blast cell count, intermediate or unfavourable cytogenetics and *FLT3* mutation		Prognostically adverse [533]
High *PRDM16* expression	*FLT3*-ITD, *NUP98-NSD1* and *KMTR1*-PTD	A quarter of childhood cases	Prognostically adverse [534]

AML, acute myeloid leukaemia; CRR, complete remission rate; DFS, disease-free survival; FAB, French–American–British (classification); MDS, myelodysplastic syndrome; OS, overall survival; RFS, relapse-free survival; WBC, white blood cell count.

Prognostic significance of cytogenetic and genetic events

Cytogenetic and molecular genetic information permits patients to be assigned to broad groups that differ in prognosis and for whom the optimal treatment will therefore differ (Tables 3.9–3.11, see also Table 2.16).

A molecular genetic classification based on a study of 1540 patients has recently identified 11 genomic categories, eight of which reiterated WHO categories but three of which were previously unidentified: (i) a poor prognosis group with mutations in genes that control RNA splicing, chromatin structure or transcription (of which the most common were *RUNX1*, *KMT2A*-PTD, *SRSF2*, *DNMT3A*, *ASXL1*, *STAG2* and *NRAS*) representing 18% of patients; (ii) a very poor prognosis group with *TP53* mutations, chromosomal aneuploidy or both representing 13% of patients; and (iii) a good prognosis group with *IDH2* R172 mutation with no other class-defining lesion, representing 1% of patients [353].

Table 3.9 Prognostic stratification using molecular analysis.

Molecular abnormality	Prognosis	Median overall survival
PML-RARA, *CEBPA* double mutated	Very favourable	Not reached
RUNX1-RUNX1T1, *CBFB-MYH11*, *NPM1* mutated with *FLT3*-ITD negative	Favourable	62.2 months
CEBPA single mutated, *FLT3*-ITD with or without *NPM1* mutation	Intermediate	25.6 months
KMT2A-PTD and/or *RUNX1* mutated and/or *ASXL1* mutated or all genes wild type	Unfavourable	13.7 months
TP53 mutated	Very unfavourable	4.6 months

ITD, internal tandem duplication; PTD, partial tandem duplication
Adapted from Grossmann *et al.* 2012 [507].

Table 3.10 Prognostic classification based on United Kingdom Medical Research Council trial data.

Favourable	t(15;17)(q24.1;q21.2) t(8;21)(q22;q22.1) inv(16)(p13.1q22)/t(16;16)(p13.1;q22)
Intermediate	Entities not classified as favourable or adverse
Adverse	Abnormal 3q excluding t(3;5)(q25;q34) inv(3)(q21.3q26.2)/t(3;3)(q21.3;q26.2) add(5q), del(5q), −5 −7, add(7q), del(7q) t(6;11)(q27;q23.3) t(10;11)(p12;q23.3) t(11q23) excluding t(9;11)(p21.3;q23.3) and t(11;19) (q23.3;p13) t(9;22)(q34.1;q11.2) −17, abnormal 17p Complex karyotype (≥4 unrelated abnormalities)

Adapted from Grimwade *et al.* 2010 [535].

Table 3.11 European LeukaemiaNet prognostic scoring system.

Risk category	Cytogenetic or molecular genetic abnormality
Favourable	t(8;21)(q22;q22.1)/*RUNX1-RUNX1T1*; inv(16)(p13.1q22) or t(16;16)(p13.1;q22)/*CBFB-MYH11* Mutated *NPM1* without *FLT3*-ITD (normal karyotype) Biallelic mutation of *CEBPA* (normal karyotype)
Intermediate-I	*FLT3*-ITD with mutated or wild-type *NPM1* (normal karyotype) Wild-type *NPM1* without *FLT3*-ITD (normal karyotype) Normal karyotype unless assigned to favourable
Intermediate-II	t(9;11)(p21.2;q23.3); *KMT2A-MLLT3* Cytogenetic abnormalities not classified as favourable or adverse
Adverse	inv(3)(q21.3q26.2) or t(3;3)(q21.3;q26.2); *MECOM*, *GATA2* t(6;9)(p23;q34.1); *DEK-NUP214* t(v;11)(v;q23.3); *KMT2A* rearranged – other than t(9;11)(p21.3;23.3) −5 or del(5q), −7 or abnormal 17p Complex karyotype*: three or more chromosomal abnormalities in the absence of t(8;21), inv(16), t(16;16), t(15;17), t(9;11), t(v;11), t(6;9), inv(3) or t(3;3)

ITD, internal tandem duplication;
* For standardized reporting it is advised that when there is a complex karyotype involvement of 5q, 7q or 17p is indicated
Adapted from Döhner *et al.* 2015 [511].

Secondary acute myeloid leukaemia

Secondary acute myeloid leukaemia (s-AML) is not a WHO category. However, in WHO terminology it refers to AML that represents transformation of MDS, MDS/MPN or MPN, and does not refer to t-AML. This is a useful distinction to make. However, sometimes it can be difficult to distinguish between s-AML and t-AML when leukaemogenic therapy, such as ^{32}P or busulfan, has been used in the treatment of an MPN. 'Blast crisis' and 'acute transformation' are alternative terms, used particularly in the case of CML.

Clinical and haematological features

There are generally no specific clinical or haematological features. Occasionally the second cytogenetic/molecular event conveys specific morphological features, as when t(15;17) occurs as a second event in CML. The prognosis is poor.

Cytogenetic and molecular genetic features

Clonal evolution is responsible for s-AML. New cytogenetic abnormalities may be present. Specific genetic events occurring before acute transformation have been identified in more than 95% of cases. Recurrent mutations are found in *SRSF2*, *SF3B1*, *U2AF1*, *ZRSR2*, *ASXL1*, *EZH2*, *BCOR* and *STAG2* [536].

Conclusions

The classification of acute myeloid leukaemia is becoming increasingly complex and this 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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4

Acute Lymphoblastic Leukaemia and Acute Leukaemia of Ambiguous Lineage

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Introduction

The diagnosis of acute lymphoblastic leukaemia (ALL) and acute leukaemia of ambiguous lineage depends on cytology and immunophenotyping. Cytogenetic and molecular genetic analysis is also of crucial importance. Cytochemistry is only relevant for identifying myeloid differentiation in the case of acute leukaemias of mixed lineage. The 2008 World Health Organization (WHO) classification and its 2016 revision recognize B-lineage and T-lineage ALL, grouping them with lymphoblastic lymphoma and designating them respectively B lymphoblastic leukaemia/lymphoma and T lymphoblastic leukaemia/lymphoma [1]. In addition, mixed phenotype acute leukaemia (previously known as biphenotypic and bilineage leukaemia) and a number of acute leukaemias of rare or ambiguous lineage are recognized [2].

Acute lymphoblastic leukaemia has a bimodal age distribution with a peak in early childhood and a rising incidence in older adults. It is more common in males than in females. In the USA, childhood ALL is considerably less common in Black Americans than in Whites, and is more common in Hispanics than in other Whites [3].

The risk of developing ALL is increased in association with constitutional chromosomal abnormalities, specifically +21c and rob(15;21)(q10;q10)c, with polymorphisms in *IKZF1*, *ARID5B*, *CEBPE*, *CDKN2A*, *CDKN2B*, *GATA3*, *PIP4K2A* and *TP63*, and with germline mutations in *TP53*, *NRAS*, *PTPN11* and, less often, *PAX5* and *ETV6* [4–7]. Environmental influences appear to be of considerable importance, delayed exposure to a common pathogen being a postulated mechanism of childhood ALL.

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. There is no clear cut-off point for blast cell numbers for a diagnosis of ALL, but 20% or more bone marrow blasts is commonly used [8]. 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 T-cell receptor (TCR) loci 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 and its 2016 revision 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 2008/2016 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.

Prognosis in ALL is indicated by presenting white cell count (WBC), age, immunophenotype, response to treatment as indicated by response to corticosteroids and minimal residual disease (MRD), and genetic subtype [9]. The better prognosis associated with B-lineage ALL is attributable to the two good prognosis entities, high hyperdiploidy and t(12;21) [9]. Within the category of Ph-negative B-lineage ALL, *KMT2A* (previously designated *MLL*) rearrangement, partial *IKZF1* deletion and a WBC of at least 30×10^9/l are prognostically adverse [10]. For B-lineage cases, prognosis can be assessed from a combination of cytogenetic and molecular genetic features. Good-risk genetic features in childhood cases include: *ETV6-RUNX1*; high hyperdiploidy; normal copy number status for nine key genes (*IKZF1*, *CDKN2A*, *CDKN2B*, *FR2* (previously *PAR1*), *BTG1*, *EBF1*, *PAX5*, *ETV6* and *RB1*); isolated deletion of *ETV6*, *PAX5* or *BTG1*; and deletion of *ETV6* with a single additional deletion of *BTG1*, *PAX5*, *CDKN2A* or *CDKN2B* [11]. All other genetic features are indicative of poor-risk disease [11]. This categorization into two risk groups was validated in an independent cohort of patients [11].

Among T-lineage cases, *NOTCH1* and *FBXW7* mutations are associated with a better prognosis, whereas mutation of *NRAS*, *KRAS* or *PTEN* is prognostically adverse [10]; a WBC of at least 100×10^9/l, central nervous system (CNS) involvement, and a pro-T/mature T immunophenotype lose their significance on multivariate analysis that considers genetic features and MRD [10].

Common clinical features in ALL include pallor, bruising, bone pain, lymphadenopathy and splenomegaly. Sometimes lytic bone lesions are present.

B lymphoblastic leukaemia/lymphoma with recurrent genetic abnormalities

B lymphoblastic leukaemia/lymphoma with 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 pre-leukaemic clone may arise during intrauterine life [12]; this may occur in the majority of cases since it was observed in 10 of 11 patients in one study [13]. The WHO classification uses the term 'hyperdiploidy' but 'high hyperdiploidy' might be preferred since cases with 47–50 chromosomes are excluded [14]. In the WHO classification the chromosomal number must be greater than 50 and is usually less than 66 [14]. Polymorphisms in *ARID5B* and *PIP4K2A* are associated with susceptibility to high hyperdiploid ALL [5] as is a germline genetic variation in *ETV6* [6].

Clinical and haematological features

This category accounts for about a quarter of childhood ALL [15,16] but only about 7–8% of ALL in adults [17,18]. 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) [19,20]. 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 French–American–British (FAB) L1 ALL. Rarely there is reactive eosinophilia [21]. Periodic acid–Schiff (PAS)-block positivity is usual. The prognosis is generally good. The best prognosis is seen with 58–66 chromosomes, then with 54–57 chromosomes, and then 51–53 chromosomes [22]. Children have a 10-year survival of more than 90% [13]. Even in adults, who have a worse prognosis than children with high hyperdiploidy, there is a 50% 5-year survival [18]. High hyperdiploidy in adults is associated with a younger age, a lower WBC and a better prognosis than other Ph-negative categories [23]. 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 [24]. The presence of unfavourable cytogenetic rearrangements, such

as t(9;22), negates the otherwise good prognosis of high hyperdiploidy [25] and such cases are not be included in this category.

The leukaemic lymphoblasts are particularly sensitive to methotrexate [26].

Immunophenotype

The immunophenotype is that of common ALL, that is CD10 is expressed whereas cytoplasmic μ chain and surface membrane immunoglobulin are not. CD19 is expressed. CD34 is often expressed whereas CD45 is weak or not expressed [27]. CD66c, which is also often expressed by Ph-positive ALL, is expressed [28]. There is strong expression of CD123 in 81% of cases, whereas normal B-cell precursors show no more than weak expression [29]. Strong expression is less common in other genetic subtypes [29].

Genetic and molecular genetic features

The term 'high hyperdiploidy' indicates that leukaemic cells have more than 50 (but usually fewer than 66) chromosomes. Cases of ALL with 'low hyperdiploidy' (47–50 chromosomes) have somewhat different characteristics, including a worse prognosis [16]. Near triploidy has a good prognosis, similar to that of high hyperdiploidy, to which it has been considered closely related [16]. The molecular mechanism of leukaemogenesis in high hyperdiploidy is unknown. The gain of chromosomes is not random, and is related to modal number [24,30]. In one study [30] 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, 4/18 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 [24]. The karyotypic abnormality can be demonstrated by conventional cytogenetic analysis (Fig. 4.1), by comparative genomic hybridization, by flow cytometry to quantitate nuclear deoxyribonucleic acid (DNA) – the DNA index – and by multicolour fluorescence *in situ* hybridization (FISH) for combinations of the more frequent supernumerary chromosomes (X, 4, 6, 8, 10, 14, 16, 18, 20, 21) [31]. Some prognostic differences have been found, related to the specific chromosomes gained [25]. 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 [25]. 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 [24], and in another gain of 11 or 17 was favourable [32]. Because of the good prognosis, screening of all patients with failed or normal cytogenetic analysis for high hyperdiploidy is

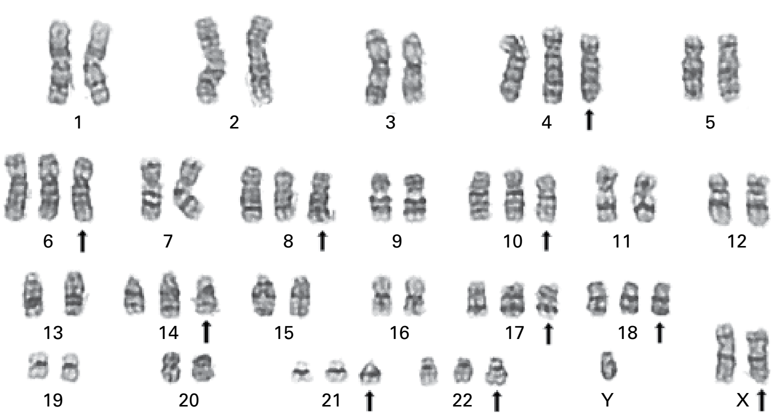


Fig. 4.1 Karyogram of a child with acute lymphoblastic leukaemia (ALL) and a high hyperdiploid clone, 56,XXY,+4,+6,+8,+10,+14,+17,+18,+21,+22; additional chromosomes are arrowed. (With thanks to Professor Lorna Secker-Walker, London, and the LRF UKCCG karyotype database.)

advised [25]. An alternative approach is to screen specifically for trisomies of 4, 10 and 17 [33,34], or for trisomies of 4 and 18 [24], since a good prognosis has been associated with these specific trisomies in different studies.

FLT3 is overexpressed [26]. Recurrent genetic abnormalities are common and include uniparental isodisomy for chromosomes 9 and 11, microdeletions of *CKDN2A*, *PAX5* and *PAN3* [9], and mutations in *NRAS*, *KRAS* and *CREBBP* [11].

B lymphoblastic leukaemia/lymphoma with t(12;21)(p13.2;q22.1); *ETV6-RUNX1* fusion

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, of similar size and have similar banding patterns. Polymorphisms in *TP63* are associated with susceptibility to *ETV6-RUNX1*-associated ALL [5].

Clinical and haematological features

When molecular techniques are used, t(12;21)(p13.2;q22.1), previously identified as t(12;21)(p12;q22), is found in 10–30% of cases of childhood B-lineage ALL [35–41] but in only 2–4% of adult cases [25,39,42]. Affected children are aged mainly between 2 and 9 years [43], and adults are usually but not always young adults [25]. When this type of leukaemia occurs in infants and children the translocation has often occurred in intrauterine life [12,44]. 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 may be about 100-fold the prevalence of this type of leukaemia during childhood. It has been suggested that the birth frequency may be much lower [45], but the evidence for a higher frequency has been vigorously defended [46,47] 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. Phagocytosis of erythrocytes and platelets by leukaemic blast cells [48] and phagocytosis of the blast cells themselves [49] have been reported. The remission rate is high. Long-term survival was initially reported to be good [40,43] or not to differ from that of ALL in general [50–52] but is now more than 90% in children [35]. Late relapses can occur, with nearly half occurring after 5 years and with relapses up to 11 years [53]. It has been suggested, on the basis of molecular evidence (analysis of TCR and IGH loci), 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 [54], with this being a postulated explanation for the observation that, unusually for ALL, there is no plateau in the event-free survival curve [53]. However, more recent investigations indicate that relapse originating in a pre-leukaemic cell is uncommon and the great majority of relapses arise in a cell of either the major leukaemic clone or a minor leukaemic subclone present at diagnosis [55]. Sensitivity to chemotherapy and the possibility of cure is retained at relapse, indicating that the mechanism of relapse is not a mutation conveying drug resistance [55]. 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 [56]. Blast cells also show *in vitro* sensitivity to doxorubicin and etoposide [57].

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. CD19 is positive. CD20 is usually not expressed. In comparison with other precursor-B ALL, there is higher expression of CD10, CD40 and human leucocyte antigen (HLA)-DR and lower expression of CD9, CD20 and CD86 [58]. There

is lack of expression of CD66c [28]. Myeloid antigens such as CD13 and CD33 are coexpressed in at least a quarter to a half of cases [40,59,60] and, conversely, t(12;21) is found in two-thirds of patients in whom these myeloid antigens are expressed [59]. The detection of CD20 expression is clinically important since the addition of rituximab in adult cases increases the effectiveness of therapy [61].

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. 2.16). A visible abnormality of chromosome 12 is present in approximately half of patients [41,62]. 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 [43,63], 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 [63]. In a significant proportion of cases, 13% in one series of patients [62], 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) [19]. Girls may lose one copy of chromosome X as a secondary cytogenetic abnormality [64]. Other cytogenetic abnormalities associated with a cryptic t(12;21) include del(6q), +8, abnormal 9p, del(11q), i(21q), +21, and translocations between chromosome 12 and a variety of partner chromosomes [60,62,65]. It is common for there to be multiple subclones at diagnosis, for example with deletion of *ETV6* or part of *RUNX1*, with duplication of *ETV6-RUNX1* or with trisomy or tetrasomy 21 [66]; such multiple subclones are seen in about a quarter of patients. Cases of ALL with near tetraploidy often have *ETV6-RUNX1* [67]. Secondary chromosomal abnormalities are not of any prognostic significance [62].

The genetic defect can be detected by reverse transcriptase polymerase chain reaction (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. 4.2) or two probes that together span *ETV6* (dual-colour, break-apart FISH technique). If *ETV6* and *RUNX1* probes or a split signal FISH technique

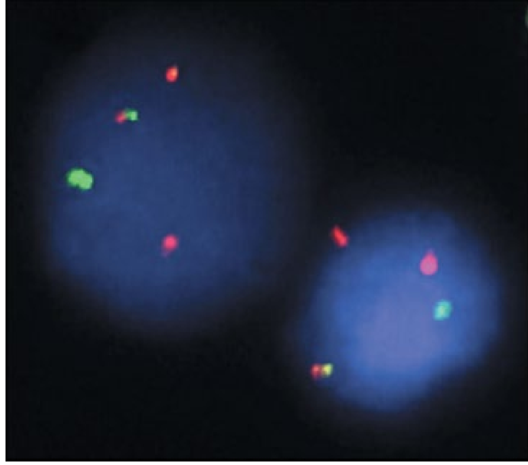


Fig. 4.2 Fluorescence *in situ* hybridization (FISH) demonstrating *ETV6-RUNX1* fusion in interphase cells of a patient with ALL. A dual-colour translocation probe has been used. The *ETV6* probe (green) binds 5′ to the 12p13.2 breakpoint. The *RUNX1* probe (red) spans the entire gene including the 21q22 breakpoint. Normal cells will thus have separate red and green signals (two of each). The leukaemic cells shown have one normal green *ETV6* signal, one large red signal (normal *RUNX1*), one smaller red signal (residual *RUNX1*) and one fused double-colour *ETV6-RUNX1* signal. (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

is used it is possible to detect not only the translocation but also the deletion of a normal *ETV6* allele (Fig. 4.3). The case detection rate is higher with RT-PCR [29]. 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% [50]. The level of MRD at the end of induction therapy, as evaluated by a limiting dilution PCR assay, is of prognostic significance [51].

Secondary genetic abnormalities include submicroscopic alterations in *EBF1*, *PAX5*, *BTLA*, *TOX*, *NR3C1*, *BMF*, *TBL1XR1* and *BTG1* [9].

B lymphoblastic leukaemia/lymphoma with t(9;22)(q34.1;q11.2); *BCR-ABL1*

Acute lymphoblastic leukaemia associated with t(9;22)(q34.1;q11.2) [68] 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 [69,70]. 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 [71]. A *GATA3* polymorphism has been linked particularly but not only to susceptibility to Ph-positive ALL in adolescents and young adults [7].

Clinical and haematological features

The prevalence of Ph-positive ALL increases steadily with increasing age [23,72] (Fig. 4.4). 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 [23,73–75]. 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 [74,76]. Rare cases have L3 cytological features [77]. Occasional cases have an increased basophil count (Fig. 4.5). There is a significant association with the presence of micromegakaryocytes but this observation is not pathognomonic of Ph positivity [78].

With conventional chemotherapy the prognosis was generally poor, although it was better in children aged between 1 and 9 years

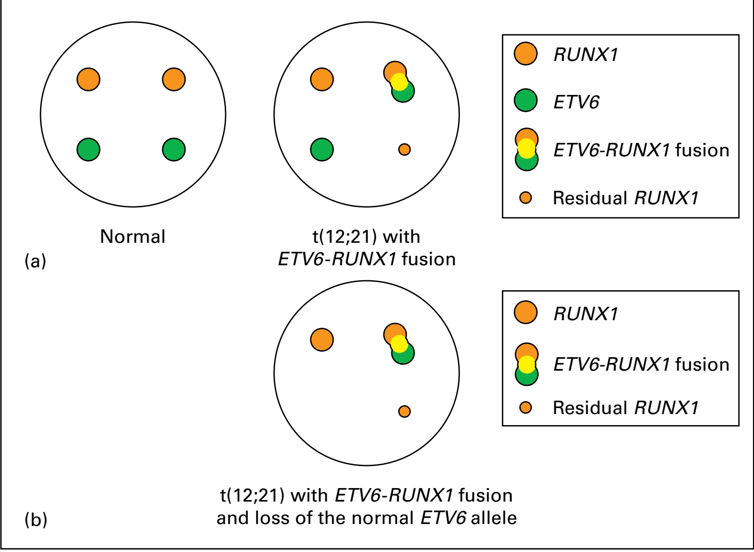


Fig. 4.3 Diagrammatic representation of extra-signal, dual-colour FISH for detection of *ETV6-RUNX1* fusion in cryptic t(12;21)(p13.2;q22.1), using a red *RUNX1* probe and a green *ETV6* probe. (a) In a normal cell there are two red signals and two green signals whereas in a cell with t(12;21) there is one red *RUNX1* signal, one green *ETV6* signal, a yellow fusion *ETV6-RUNX1* signal and an extra small red 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.

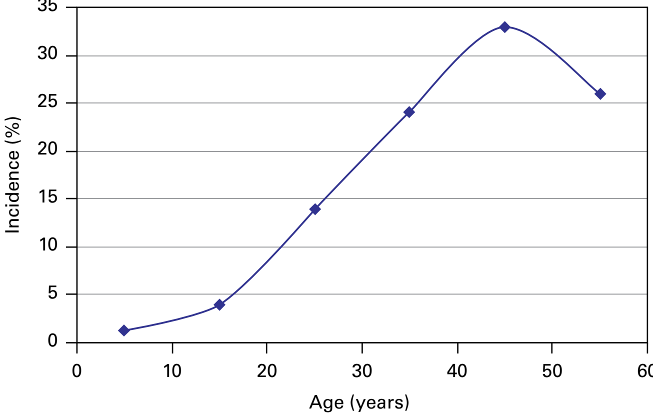


Fig. 4.4 Graph showing the rising incidence of Ph-positive ALL with age.

than in adolescents, and better in adolescents than in adults. Event-free survivals were 30–50% in children [53,79] and 15–20% in adults [62,80]. Allogeneic stem cell transplantation therefore became the treatment of choice in adults [80].

The advent of tyrosine kinase inhibitors led to a marked improvement in prognosis, for example 58% 2-year overall survival in a series with frequent use of haemopoietic stem cell transplantation in addition to imatinib-containing chemotherapy [81], and a 71% 7-year event-free survival in children treated with imatinib and intensive chemotherapy without transplantation [9]. There is no clear benefit in allogeneic transplantation in children when imatinib is combined with chemotherapy [82]. Imatinib plus corticosteroids is of benefit in elderly patients who, in one study, achieved a median survival of 20 months [83]. With later generation tyrosine kinase inhibitors, results are better than with imatinib; a trial of chemotherapy plus ponatinib achieved a 2-year event-free survival of 81% [84]. The most usual treatment is now chemotherapy plus a tyrosine kinase inhibitor without transplantation in children and with transplantation in adults.

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 [17,74,85–87]. CD19 is expressed and usually terminal deoxynucleotidyl transferase (TdT) but CD117 is usually negative [68]. In two series of patients, CD34 was more often expressed than in ALL in general [76,88], but this was not so in another series [74]. In various series of patients, the frequency of detection of myeloid antigens such as CD13 and CD33 has ranged from 20% to 75% [16,74,86,89]. The myeloid antigen, CD66c, is more often expressed in *BCR-ABL1*-positive ALL, 82% (14/17) in comparison with 31% (24/77) of *BCR-ABL1*-negative cases [90]. CD25 is often expressed [91]. 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 [92]. Monoclonal antibodies that detect Ph-positive cells with a high sensitivity have been produced [93]; these have been found to be directed at CD66c, which is also expressed in ALL associated with high hyperdiploidy [28].

Cytogenetic and molecular genetic features

t(9;22) is also the characteristic cytogenetic abnormality of chronic myeloid leukaemia (CML). The translocations in ALL and CML do not differ cytogenetically but at a molecular level the breakpoint on chromosome 22 may differ. The mechanism of leukaemogenesis is

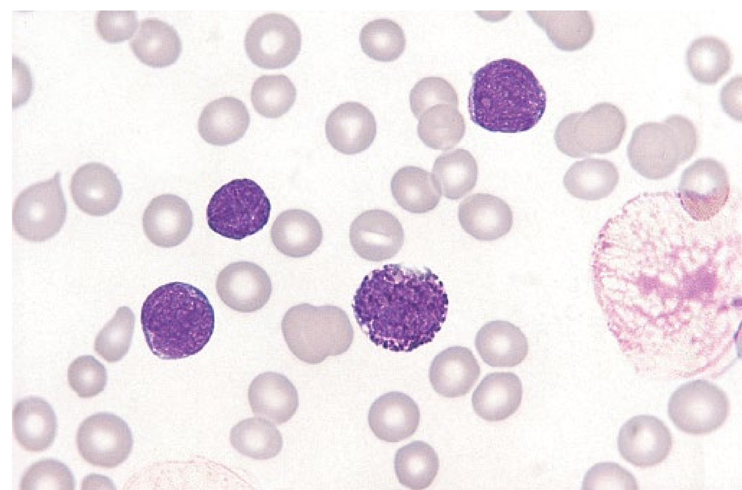
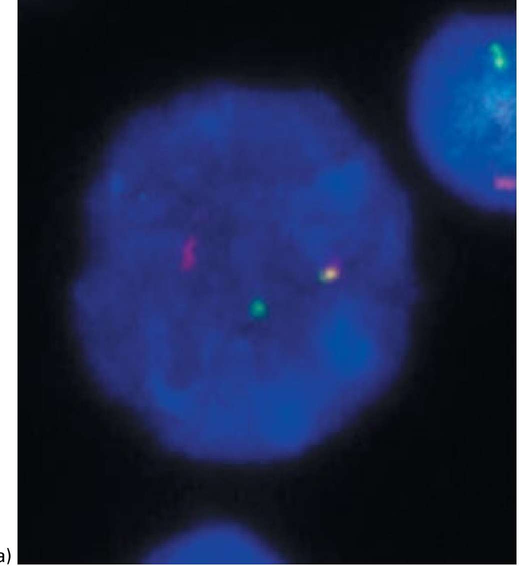


Fig. 4.5 Peripheral blood (PB) film from a patient with French–American–British (FAB) L1 Ph-positive ALL showing blast cells and a basophil. May–Grünwald–Giemsa (MGG) × 100.

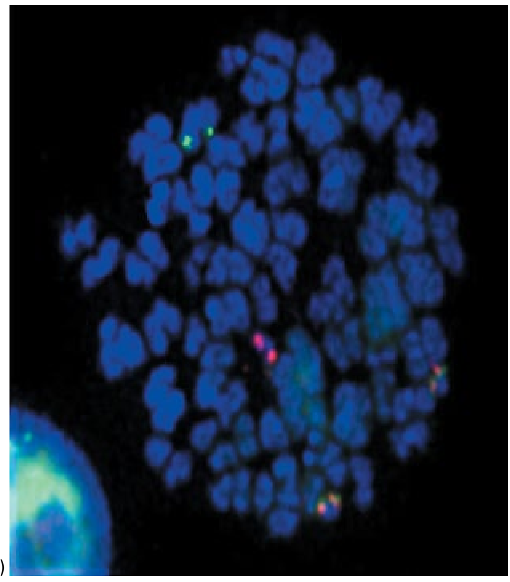
fusion of part of the *ABL1* oncogene from chromosome 9 with part of the *BCR* gene on chromosome 22 to form a hybrid gene on chromosome 22 designated *BCR-ABL1. ABL1* is homologous with v-*abl*, a retroviral oncogene, which has a role in murine leukaemia. *BCR-ABL1* encodes a chimeric protein with aberrant tyrosine kinase activity, which functions in intracellular signalling pathways. A minority of patients with Ph-positive ALL (about one-third), have a major breakpoint cluster region (M-BCR) breakpoint, as in CML, and the BCR-ABL1 protein has a molecular weight of 210 kDa. In the majority of cases (70–75%), the breakpoint is in the minor BCR (m-BCR) breakpoint on chromosome 22, and the BCR-ABL1 protein has a molecular weight of 190 kDa; this breakpoint is very rare in CML. In less than 1% of Ph-positive patients, both transcripts are present [87]. There are only trivial haematological differences between patients with p210 and p190; expression of CD34 may be more frequent in the latter group [94]. Prognosis was better in those with an M-BCR breakpoint (p210) in one study [80] but not in another [23]. The prevalence of the two molecular variants varies with age, with cases with a 190 kDa transcript increasing from adolescence and those with a 210 kDa transcript from early adult life [87].

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) [41], abnormalities of 9p (e.g. del(9p), often with loss of the *ABL1-BCR* gene), trisomy 21 and high hyperdiploidy [18,73,81,86,95] (Fig. 4.6). Secondary abnormalities seen in 10–20% of patients are monosomy 7, trisomy 8 and plus X [81,95]. A complex karyotype (at least two extra abnormalities) was seen in half the patients in one study [95]. The presence of secondary cytogenetic abnormalities and of a complex karyotype is associated with a worse prognosis [81,95]. Monosomy 7 as a secondary abnormality is associated with an M-BCR breakpoint, with coexpression of myeloid antigens, and has been associated with a particularly bad prognosis. Secondary abnormalities of 9p are associated with m-BCR breakpoints, with lack of expression of myeloid antigens and in the past with a very bad prognosis. The prognosis appears to be somewhat better in cases with hyperdiploidy or + der(21) as an associated abnormality [73]. The prognostic significance of secondary cytogenetic abnormalities is likely to have lessened with the advent of tyrosine kinase therapy.

The cytogenetic defect is detectable by a variety of FISH techniques (see page 378). The



(a)



(b)

Fig. 4.6 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. (With thanks to Dr Magda Jabbar Al-Obaidi, London.)

molecular defect can be detected by RT-PCR. FISH studies show that, as for CML, 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 CML; this loss of chromosomal material can be seen in patients with an m-BCR as well as in those with an M-BCR breakpoint [96]. FISH permits the detection of *BCR-ABL1* fusion with a normal karyotype (representing about a sixth of cases) and also the rare cases due to insertion of *ABL1* into *BCR* [41]. RT-PCR can be used to monitor MRD, the detection of which is of prognostic significance [97]. Detection of the BCR-ABL1 protein by flow cytometry using a commercially available bead assay permits rapid diagnosis [98].

PAX5 deletion occurs as a secondary genetic event in about half of patients. An even more common second genetic defect is deletion or mutation of the *IKZF1* gene, encoding the lymphoid transcription factor, IKAROS [99]. *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. There may also be mutation of *CDKN2A* and *CDKN2B* [11].

B lymphoblastic leukaemia/lymphoma with t(4;11)(q21.3;q23.3) or other translocation with an 11q23.3 breakpoint; *KMT2A-AFF1* (previously *MLL-MLLT2*) or other fusion gene incorporating *KMT2A*

In the WHO classification this subtype of ALL includes cases with t(4;11) and those with other translocations [100]. These two groups differ in some characteristics. *KMT2A* has more than 100 partner genes. ALL with t(4;11)(q21.3;q23.3) occurs at all ages but is particularly frequent among babies with congenital ALL and in young infants [101,102]. It constitutes more than half of these cases. Cases occurring in infants often

originate in intrauterine life [12,103]; exposure to topoisomerase II-interactive agents, such as dietary bioflavinoids, during gestation is suspected as an aetiological factor. One percent of children with ALL above the age of 1 year have t(4;11) [25] whereas in adult ALL the prevalence is 3–5% and increases with age [18,85].

Other translocations with 11q23.3 breakpoints and rearrangement of the *KMT2A* gene are also associated with B-lineage ALL and, sometimes, with mixed phenotype acute leukaemia (MPAL) or acute myeloid leukaemia (AML). Some of these are shown in Tables.3.3 and 3.4. Among adults these cases comprise about 4% of cases of ALL [18] and in children 2–3% [16]. Although leukaemias associated with these translocations have some features in common they differ in other characteristics.

Clinical and haematological features

In patients with t(4;11), marked splenomegaly and a high WBC are common, as is CNS 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. Age less than 6 months, a WBC greater than 300×10^9/l and CNS infiltration are prognostically adverse [104]. In a UK series, the 5-year event-free survival in children with t(4;11) or other translocations involving 11q23 was 30% [105]. In a Japanese series, the 3-year event-free survival was 34% [106]. 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 [107]. The survival of infants with 11q23.3 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 *KMT2A* rearrangements [106,108], or at least no worse than when there is t(9;11)(p21.3;q23.3) or t(11;19)(q23.3;p13) [107]. In older children, t(4;11) and t(9;11) are associated with worse outcome than other 11q23.3 rearrangements [108]. In adults, t(4;11) is associated with an adverse prognosis [23], but this is not so of patients with other 11q23.3 breakpoints, such as t(11;19) [23]. *KMT2A*-rearranged ALL is particularly responsive to cytarabine [109].

The clinical features of cases with variant translocations 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 CNS disease. One neonate with congenital leukaemia associated with t(11;19)(q23;p13) has been reported [110]. If the *KMT2A* gene is rearranged the prognosis is equally poor in all translocations, with an 11q23.3 breakpoint in some [16,106,111] but not all [108] series. Pui *et al.* found an equally dismal prognosis with all 11q23.3 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 [108]. 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 [112].

Immunophenotype

t(4;11)(q21.3;q23.3) is strongly associated with early-B-precursor (pro-B) ALL, that is 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 [113]. CD1d may be expressed [114]. Myeloid antigens are expressed in about half of cases [17]. Myeloperoxidase (MPO) messenger ribonucleic acid (mRNA) and protein may be expressed [115]. At relapse the immunophenotype is sometimes that of MPAL or of acute monoblastic leukaemia. Positivity with a monoclonal antibody (McAb) to chondroitin sulphate proteoglycan, NG2, has been demonstrated [113]. CD133 was consistently expressed in a small number of patients tested, whereas it

was expressed in less than half of other patients with ALL [116].

The immunophenotype associated with variant translocations is usually also that of pro-B ALL. Positive reactions with NG2 are observed in cases with t(11;19) as well as cases with t(4;11) [113]. Cases with t(11;19)(q23;p13) may likewise show aberrant expression of CD15 and CD65 [113]. The combination of homogeneous expression of CD4, expression of CD56 and lack of expression of CD34 shows a significant correlation with a rearranged *KMT2A* gene [117].

Cytogenetic and molecular genetic features

The molecular mechanism of leukaemogenesis in association with t(4;11)(q21.3;q23.3) (Fig. 4.7) is formation of the fusion genes, *KMT2A-AFF1* and *AFF1-KMT2A*, the former incorporating part of the *KMT2A* (previously known as *MLL*, myeloid–lymphoid leukaemia) gene at 11q23.3 and part of the *AFF1* gene (previously known as *AF4* then *MLLT2*) from 4q21.3 [118]. *AFF1* encodes a protein that is probably a transcription factor [119]. Both fusion proteins have been shown to have oncogenic potential and both are generally present [120]. However, *KMT2A-AFF1* appears to be essential for leukaemogenesis whereas *AFF1-KMT2A* may be dispensable [121]. Secondary genetic abnormalities are less common than in other types of ALL [100]; they include mutation of *CDKN2A* and *CDKN2B*, *NRAS*, *KRAS* and *FLT3* [11]. *FLT3* is overexpressed in cases with t(4;11) and with variant translocations [26]. Prognosis in *KMT2A*-rearranged infant ALL is worse if there is high level expression of *FLT3* [122]. *RAS* gene mutations occur in about a quarter of patients and are associated with worse prognosis [123]. Low expression of *HOXA* cluster of genes is prognostically adverse [104].

In variant translocations, the molecular mechanism is the fusion of part of the *KMT2A* 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, 11, 12, 19, 20 and X [118,124–127]. Of cases of ALL with *KMT2A* rearrangement, 94% are accounted for by six rearrangements: t(4;11) with *AFF1* involvement, t(11;19) with *MLLT1*, t(9;11) with *MLLT3*, t(10;11) with *MLLT10*, t(6;11) with *AFDN* (previously *MLLT4*) and t(1;11) with *EPS15* [127]. Cases that have cytogenetic abnormalities with 11q23

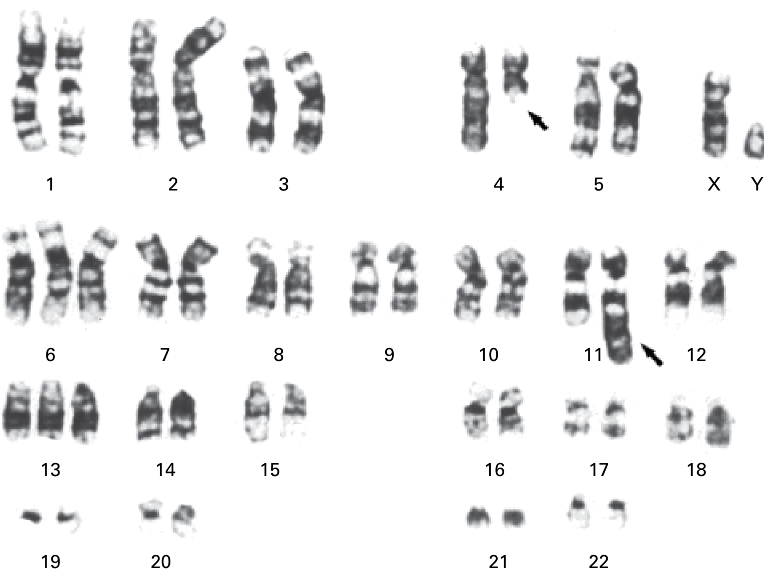


Fig. 4.7 A karyogram showing t(4;11)(q21.3;q23.3). (With thanks to the late Professor Harry Smith.)

breakpoints but without *KMT2A* rearrangement, for example many cases with del(11)(q23), have different disease characteristics including a better prognosis and are not included in this subtype of ALL [111].

Rearrangement of the *KMT2A* gene, including that of t(4;11), can be detected by dual-colour, break-apart FISH with a 5′ *KMT2A*–3′ *KMT2A* probe. In about a third of patients with 11q23.3 translocations there is loss of 3′ *KMT2A* and therefore loss of one colour of the signal. *KMT2A-AFF1* fusion can be detected by RT-PCR. MRD, which is of prognostic significance, can be monitored by RT-PCR [97]. Rapid reversion to negativity and persistence of negativity for more than 3 months is predictive of a better outcome [128]. Only about 50% of patients have rearrangements of antigen-receptor genes and such rearrangements are therefore often not useful for MRD monitoring [129].

Whole chromosome paints (Fig. 4.8) can be useful for the detection of variant translocations. Many of the alternative molecular rearrangements resulting from translocations with an 11q23.3 breakpoint can be demonstrated by RT-PCR.

B lymphoblastic leukaemia/lymphoma with t(1;19)(q23;p13.3); *TCF3-PBX1*

This subtype constitutes 2–5% of childhood ALL and 1–3% of adult cases [16,18,85]. The translocation was first recognized by Carroll and colleagues in 1984 [130]. A minority of childhood cases are of intrauterine origin [12,131].

Clinical and haematological features

Adult patients are relatively young [16,132]. In one series there was a correlation with a high WBC, non-White ethnic origin and CNS disease [133]. However, in other series of adult cases the WBC was generally low [17,18]. Cytological features are most often FAB L1 but sometimes L2 and sometimes L3 [134] (Fig. 4.9). The cytological features can change from L1 to L3 with disease progression [135]. The prognosis in childhood cases was previously poor. 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 [16,53,136]. However, a Nordic trial found that although event-free survival was similar, overall survival was shorter than in patients with t(12;21) or high hyperdiploidy [137]. A poor prognosis has been reported in adults [138]. Because of the greater risk of CNS disease and the biological features of the leukaemic cells, an increased dose of methotrexate may be indicated [139]. Leucoencephalopathy occurring during complete remission has been reported in a number of studies to be more common in this subtype of ALL [140].

Immunophenotype

The immunophenotype is often that of pre-B ALL, that is 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 [133] or there may be both positive and negative blast cells. CD45 is strongly expressed. This immunophenotype is characteristic but not specific [28]. 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 [141]. Strong CD9 and absent or weak CD34 expression is also suggestive of this type of ALL [132]. Polyclonal [142] and monoclonal [143] 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 [143]. On immunohistochemistry there is expression of BCL6, with other types of B-ALL being weak or negative [144]. Other germinal centre

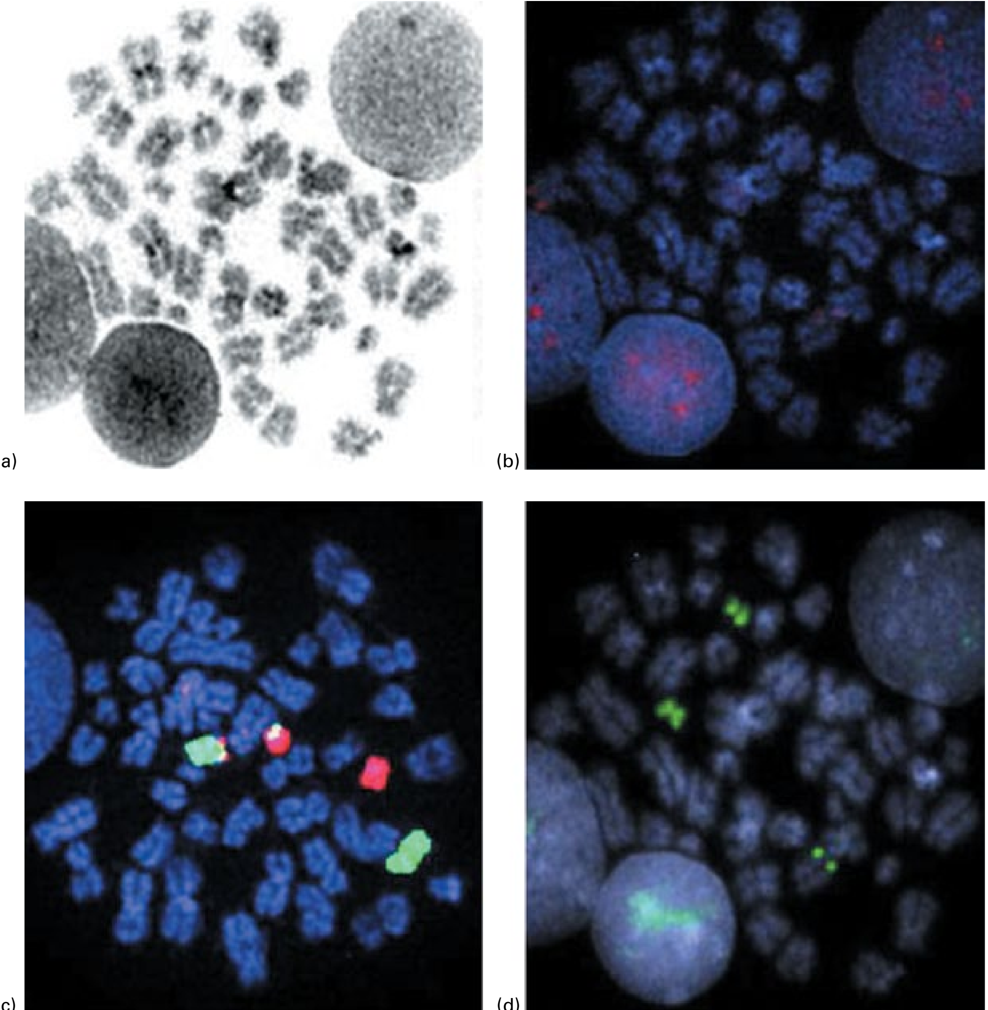


Fig. 4.8 A subtle t(11;19)(q23.3;p13.3) demonstrated by FISH: (a) metaphase spread; (b) rearrangement of *KMT2A* demonstrated with a *KMT2A* probe (red) which has been split so that there are three signals instead of two; (c) whole chromosome paints for chromosome 11 (green) and chromosome 19 (red) showing 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. (With thanks to Dr Magda Jabbar Al-Obaidi.)

lymphocyte antigens, LMO2 and HGAL, are also often expressed [144].

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 19p13.3 to form a hybrid *TCF3-PBX1* gene, which encodes an abnormal transcription factor [145]. There is also aberrant expression of *MERTK*, which encodes a receptor tyrosine kinase [146]. ZAP70 is overexpressed, as it is in other cases of ALL with a pre-B immunophenotype [147].

t(1;19) may occur as a balanced or unbalanced translocation. A straightforward balanced translocation, t(1;19)(q23;p13.3), is the less common abnormality. More common is the

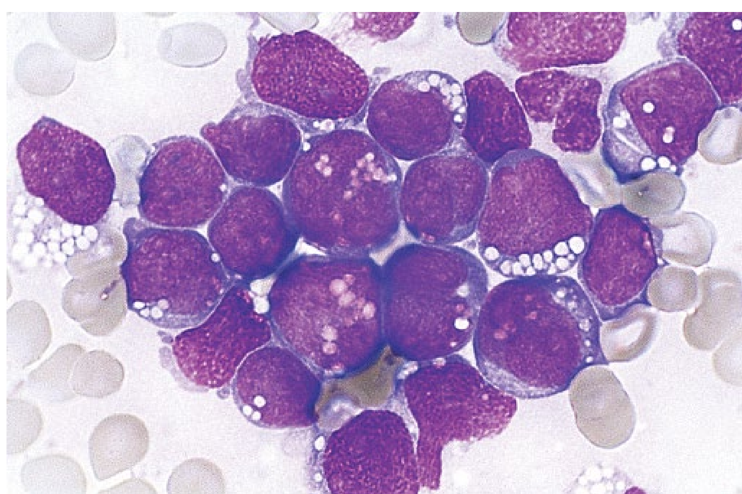


Fig. 4.9 Bone marrow (BM) film in ALL with t(1;19)(q23;p13.3) and FAB L3 cytological features. MGG × 100. (With thanks to Dr Georges Flandrin, Paris.)

unbalanced der(19)t(1;19)(q23;p13.3) in which the derivative chromosome 1 has been lost and has been replaced by a second copy of the normal chromosome 1 (Fig. 4.10). A better prognosis in patients with an unbalanced translocation was found in three series of patients, [112,136,148] but not in two others [137,149].

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. It has been associated with disseminated intravascular coagulation (present in about 60%) and hypercalcaemia (present in about 70% and mediated in part by parathyroid hormone-related protein) [150]. The prognosis is very poor [9] and this should be considered as an entity separate from ALL with t(1;19).

A double-colour, break-apart FISH strategy has been devised that both detects t(1;19) and distinguishes balanced and unbalanced translocations [151]. In addition, this FISH technique gives abnormal results 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 [128].

Secondary genetic abnormalities include mutations of *TCF3*, *PAX5*, *CDKN2A* and *CDKN2B* [11].

B lymphoblastic leukaemia/lymphoma with t(5;14)(q31.1;q32.1); *IGH/IL3*

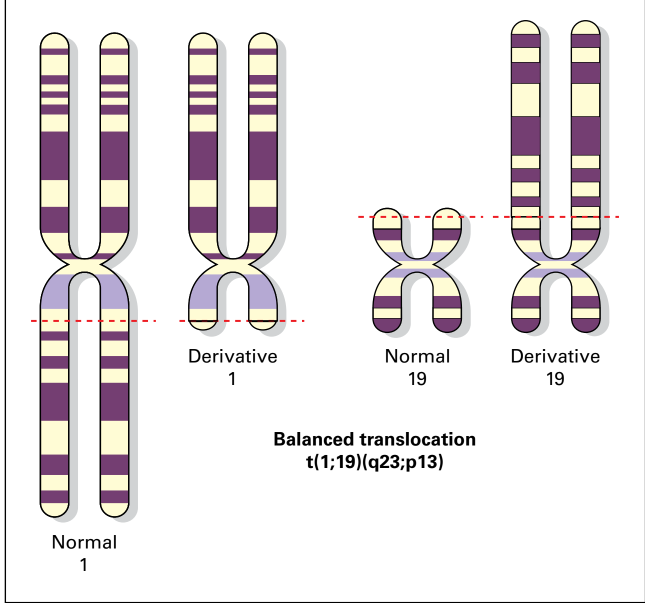
This is a rare form of B-lineage ALL (<1% of cases) but is important because it may be confused with eosinophilic leukaemia if not adequately investigated.

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 [152] (Fig. 4.11). Because of the large number of eosinophils and precursors in the bone marrow, the blast percentage may not be very high and no specific blast percentage is required for this diagnosis.

Immunophenotype

The immunophenotype is that of common ALL, CD19 and CD10 being expressed.



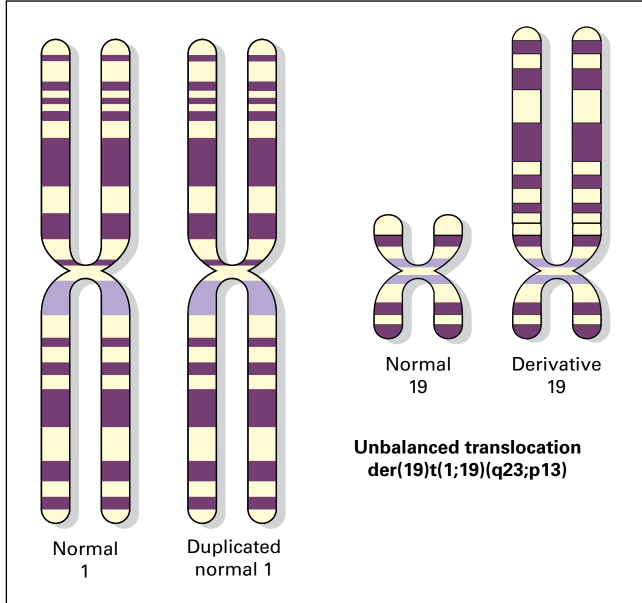


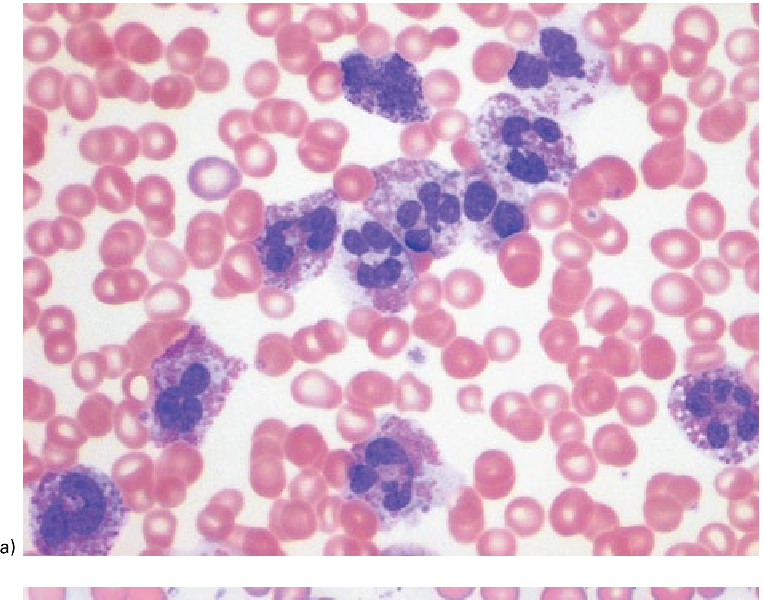
Fig. 4.10 Diagrammatic representation of balanced and unbalanced forms of t(1;19)(q23;p13.3).

Cytogenetic and molecular genetic features

The mechanism of eosinophilia is dysregulation of *IL3* leading to high levels of interleukin (IL) 3. 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** or 24–31


(a)

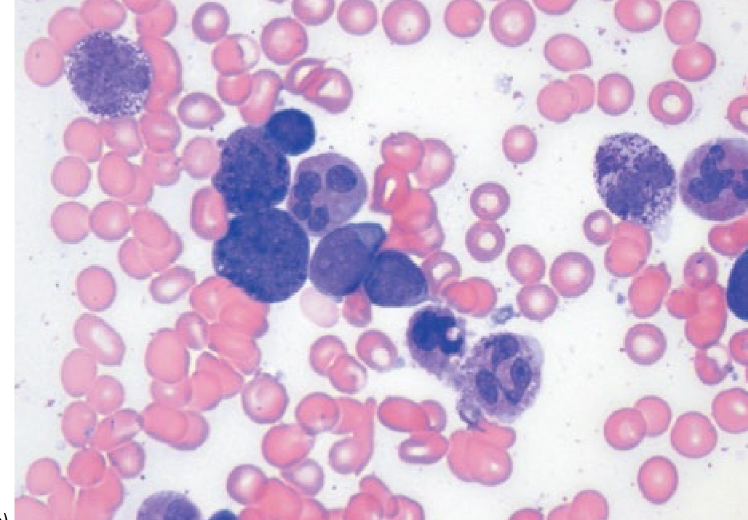

(b)

Fig. 4.11 ALL with eosinophilia associated with t(5;14)(q31.1;q32.1). (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. (With thanks to Dr James Vardiman, Chicago.)

chromosomes), low hypodiploidy (**30–39** or **33–39** chromosomes) and high hypodiploidy (**40–43** or 42–45 chromosomes), the WHO categorization being shown in bold [153,154]. Near diploid (44 or 45 chromosomes) is a distinct entity and can reasonably be excluded from the high hypodiploid category [4]. In the WHO classification hypodiploidy is defined by having fewer than 46 chromosomes but with the comment that it might be better defined by fewer than 45 or fewer than 44 chromosomes [155]. Near haploid cases may be confined to children [154]. Near haploid metaphases usually retain both sex chromosomes and preferentially

gain chromosomes 10, 14, 18 and 21 into the haploid chromosome set [155]. Low hypodiploidy and near triploidy are considered to represent the same subgroup, and subclones of both types may coexist [156]. Loss of *TP53* function may be found in association with low hypodiploidy in children [157]. 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 [153]. Both tend to have a low WBC (less than 50×10^9/l). Both near haploidy and low hypodiploidy may have an associated near diploid or hyperdiploid clone as a result of endoreduplication, and this clone may dominate; care is therefore needed in making the important distinction from diploid or high hyperdiploidy. A considerably worse prognosis than is seen in ALL in general has been reported in hypodiploidy with 33–44 chromosomes [158], in near haploidy (whether defined as 23–29 or as 24–28 chromosomes) [52,153,158], in patients with 33–39 chromosomes [153] and in patients with 30–39 chromosomes [156]. In one study, patients with more than 40 chromosomes – including adults as well as children – appeared to have an intermediate prognosis [153]. 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 [159] and thus can reasonably be excluded from this category. Such cases can result from monosomy or from unbalanced translocations leading to formation of dicentric chromosomes, particularly dic(9;20)(p11–p13;q11). *ETV6-RUNX1* may be present [4], and such cases should be assigned to that category.

Except for those with 44 or 45 chromosomes, prognosis is poor.

Clinical and haematological features

There are no specific features recognized.

Immunophenotype

The immunophenotype is usually that of common ALL with expression of CD19 and CD10.

Cytogenetic and molecular genetic features

Because of the poor prognosis, screening of all patients with failed or normal cytogenetic analysis for near haploidy and for low hypodiploidy is advised [153,155]. The DNA index is low. DNA quantification histograms also permit detection of a prognostically important small hypodiploid clone.

There may be associated structural chromosomal abnormalities as well as associated molecular changes.

Near-haploid ALL is characterized by mutations in *NF1*, *NRAS*, *KRAS* and *PTPN11* and inactivation of *IKZF3*, all leading to RAS activation [4,160]. Low hypodiploidy is associated with a familial germline *TP53* mutation, reported in half to more than 90% of cases, suggesting that this may be a manifestation of the Li–Fraumeni syndrome [9,160]. There is also an association with deletions of *CDKN2A*, *CDKN2B* and *RB1* and inactivation of *IKZF2* [9,160].

Ph-like or *BCR-ABL1*-like B lymphoblastic leukaemia/lymphoma

This category represents about 10–13% of childhood B-lineage ALL, about 21% of adolescent cases and about 27% of young adult cases [161], with the peak incidence being in adolescents and young adults (16–40 years) [162]. It is a provisional entity in the 2016 revision of the WHO classification, under the designation *BCR-ABL1*-like lymphoblastic leukaemia/lymphoma [163]. The category was initially identified by a gene expression profile that resembled that of *BCR-ABL1*-positive ALL and subsequently was found to be characterized by activating mutations of genes encoding various kinases, cytokines and cytokine receptors. With standard chemotherapy, prognosis is adverse whereas responsiveness to appropriate kinase inhibitors is likely, making the detection of this subtype of ALL important [161]. Polymorphisms in *GATA3* are associated with increased susceptibility to Ph-like ALL [5]. Down syndrome predisposes to a particular

molecular subset, *CRLF2* rearrangement being seen also in more than 50% of cases of ALL in Down syndrome.

Clinical and haematological features

Patients are more often male and the WBC is higher than in other cases of B-lineage ALL.

Immunophenotype

The immunophenotype is usually that of common ALL; overexpression of *CRLF2* can be useful to indicate cases with upregulation of this gene [163].

Cytogenetic and molecular genetic features

BCR-ABL1-like ALL has been recognized by gene expression profiling. Underlying genetic abnormalities include dysregulation of *CRLF2* resulting from cryptic *P2RY8-CRLF2* or *IGH/CRLF2*, found in about half of cases, and in half of these cases there is also a mutation in *JAK1* or *JAK2* [9]. There are other cases resulting from translocations that involve *ABL1* or *PDGFRB*, which are likely to be responsive to tyrosine kinase inhibitors: *NUP214-ABL1* and *EBF1-PDGFRB*, *SSBP2-PDGFRB*, *TNIP1-PDGFRB*, *ZEB2-PDGFRB* and *ATF7IP-PDGFRB* [161,164]. Also described are translocations involving *EPOR* or *JAK2*, activating mutations of *FLT3*, *JAK1*, *JAK2*, *JAK3* or *IL7R* and deletion of *SH2B3* (a negative regulator of the JAK-STAT pathway) [4,161,165,166]. In total, at least 16 kinase or cytokine receptor genes have been implicated: *ABL1*, *ABL2*, *CSF1R*, *PDGFRB*, *CRLF2*, *DGKH*, *EPOR*, *IL2RB*, *IL7R*, *JAK1*, *JAK2*, *JAK3*, *NTRK3*, *PTK2B*, *TSLP* and *TYK2* [4,161,164]. More than 30 partner genes have been identified. Another group of cases have mutations in genes involving RAS signalling: *NRAS*, *KRAS*, *PTPN11* and *NF1*. *IKZF1* is mutated or deleted in two-thirds to four-fifths of cases [9,161]. *CDKN2A* and *CDKN2B* may be deleted [11].

Responsible deletions and translocations may be either cryptic or detectable on cytogenetic analysis. *CRLF2* dysregulation may be the result of a cryptic deletion at Xp22.22 or Yp11.32 bringing the gene under the influence of the *P2RY8* promoter [167]. The translocations t(X;14)(p22;q32) and t(Y;14)(p11;q32) dysregulate *CRLF2* by proximity to IGH [167], and t(14;19)(q32;p13) similarly dysregulates *EPOR* [168]. There may also be chromosomal abnormalities unrelated to the molecular changes in addition to the causative rearrangement, for example del(5)(q33q33) in the case of *EBF1-PDGFRB* [169] and cryptic t(5;12)(q33;p13) in the case of *ATF7IP-PDGFRB* [164].

B lymphoblastic leukaemia/lymphoma with iAMP21

An intrachromosomal amplification of part of chromosome 21, designated iAMP21, occurs in up to 2% of precursor B-ALL. It occurs particularly in older children (median age 9 years) [166,170]. In the 2016 revision of the WHO classification, this provisional entity incorporates cases with five or more copies of *RUNX1* (which is located within the amplified region) or with three or more extra copies of an abnormal chromosome 21 [171]. The incidence is increased more than 1000-fold in children with the rare constitutional Robertsonian rearrangement, rob(15;21)(q10;q10)c [172].

Clinical and haematological features

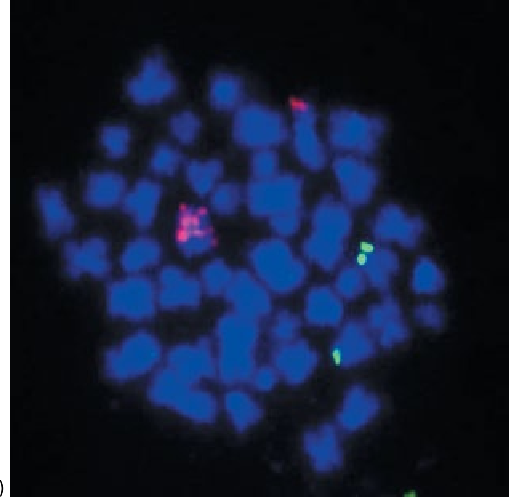
The WBC is relatively low. Outcome is poor if treated as standard risk but considerably improved if treated as high risk.

Immunophenotype

No specific immunophenotype has been identified. CD7 is often expressed [173].

Cytogenetic and molecular genetic features

FISH using a *RUNX1* probe is useful in diagnosis although the gene is not involved in pathogenesis (Fig. 4.12). Associated cytogenetic abnormalities can include gain of an X chromosome and abnormalities of chromosome 7 [171]. Common associated molecular genetic abnormalities include deletion of *RB1* or *ETV6* and mutation of *RAS* genes, *FLT3* or *CRLF2* [11,166,171].


(a)

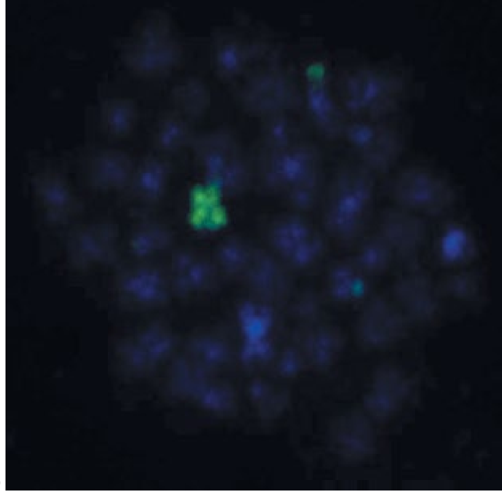

(b)

Fig. 4.12 Demonstration of internal amplification of chromosome 21q sequences (iAMP21) demonstrated by FISH: (a) using probes for *RUNX1* (red) 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. (With thanks to Dr Magda Jabbar Al-Obaidi.)

B lymphoblastic leukaemia/lymphoma, not otherwise specified

This category includes all cases of B-lineage ALL/lymphoblastic lymphoma that have not been assigned to a definitive or provisional category in the WHO classification [8]. 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. Reactive eosinophilia can occur and was described, for example, in patients with homozygous del(9)(p21), t(7;12)(q22;p13), t(3;6)(p22;p24) and a normal karyotype respectively [174,175].

B-lineage ALL is 10–20 times more common in children with Down syndrome. Prognosis is worse than in other children with ALL, mainly because of excess treatment-related mortality [176] but is improved if less intensive treatment is used.

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 abnormalities

Some patients have non-specific clonal abnormalities such as del(6q), del(9p) or del(12p). Complex karyotypes (five or more aberrations), seen in about 5% of adult patients, are associated with a worse prognosis [156].

Others have recurrent genetic abnormalities, for example dic(9;12)(p13.22;p13.22), dic(9;20)(p13;q11), t(17;19)(q22;p13.3) and a number of translocations that dysregulate genes by proximity to IGH; genes dysregulated include *ID4*,

CEBPA, *CEBPB*, *CEBPE*, *CEBPD*, *CEBPG*, *BCL2* and the microRNA *MIR125B* [166,177,178]. The t(14;18)(q32.3;q21.3) translocation that characterizes follicular lymphoma, leading to *BCL2* dysregulation, can also occur in B-lineage neoplasms with blastic morphology (FAB L1, L2 or L3) [179,180]; 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 [181] are probably better regarded as non-Hodgkin lymphoma. Rarely, a lymphoblastic transformation occurs in follicular lymphoma [182].

Cases with t(8;14)(q24.2;q32.3) with IGH/*MYC*, t(8;22)(q24.2;q11.2) with IGL/*MYC* and t(2;8)(p11.2;q24.2) with IGK/*MYC* may have a mature B immunophenotype and are classified as Burkitt lymphoma. However, some such patients (8/16 included in an ALL trial) have a pre-B or common ALL immunophenotype [23]. As their prognosis is poor if treated as ALL, it seems more appropriate that they also should be classified and treated as Burkitt lymphoma.

B-ALL in children with Down syndrome has specific genetic characteristics. Some cases fall into the *BCR-ABL1*-like category (see above) but there is also an association of Down syndrome with + X as an isolated acquired abnormality, and with t(8;14)(q11.2;q32.3) with dysregulation of *CEBPD* by proximity to the IGH locus [25].

A wide range of molecular abnormalities are seen. Deletion of *PAX5* is common. Other genes that may be deleted include genes involved in B-cell differentiation (*RAG1*, *RAG2*, *CD200* and *VPREB1* – previously *VPREB)*, transcriptional co-regulators (*BTG1*, *ETV6*, *ERG* and *TBL1XR1*) and others (*TCF3*, *EBF1*, *LEF1*, *RB1*, *CDKN2A*, *CDKN2B* and *IKZF3*) [166,183]. Sequence mutations or deletions associated with relapse can involve *TP53*, *CREBBP* and *NT5C*2 [166]. High expression of *BAALC* is prognostically adverse, independently of prognostically adverse cytogenetic rearrangements [184].

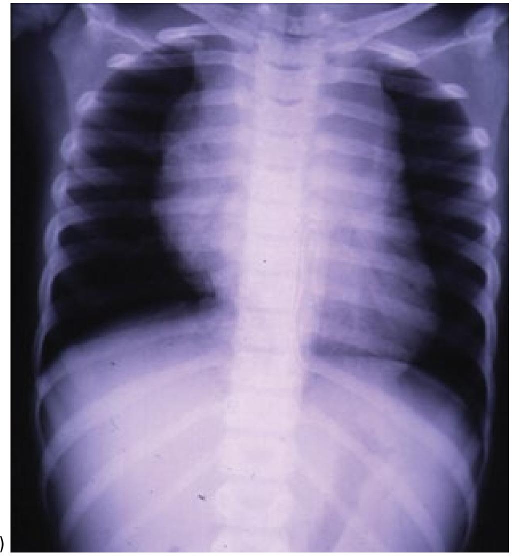
T lymphoblastic leukaemia/lymphoma

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, in adolescents rather than younger children, and constitutes a quarter of adult cases of ALL [185]. There is a male predominance. There is only a weak relationship between immunophenotype and specific cytogenetic abnormalities. One provisional specific entity, early T-cell precursor lymphoblastic leukaemia, has been recognized in the 2016 revision of the WHO [186], but otherwise T-lineage cases are not further categorized.

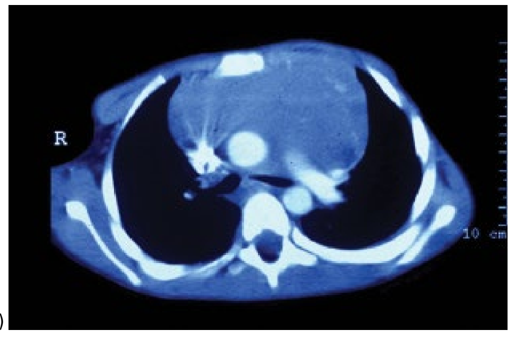
Clinical and haematological features

Primary involvement may be of the bone marrow and blood or of the thymus (Fig. 4.13) or other extranodal site. Central nervous system disease is more common than in B lymphoblastic leukaemia/lymphoma, 9% in comparison with 4% in one series of adult patients [187]. Other extranodal sites that may be involved include the liver, spleen, skin, tonsils and testes. 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 [188].

The WBC is typically high but, in comparison with B-lineage disease, bone marrow function is often better preserved. Nuclei may be hyperchromatic and irregular or convoluted (Fig. 4.14). Rarely, erythrophagocytosis by leukaemic cells has been observed [189]. 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



(a)



(b)

Fig. 4.13 Chest radiograph (a) and computed tomography scan (b) of a child with T-ALL showing massive thymic enlargement.

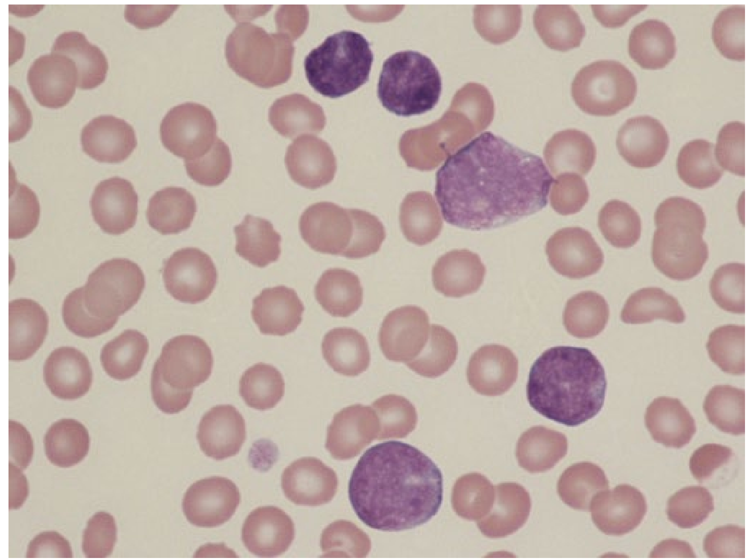


Fig. 4.14 PB film of a patient with T-ALL with a cortical T phenotype. There are five blast cells, two of which have hyperchromatic convoluted nuclei.

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 page 97. Immunophenotype is of some prognostic significance, with the cortical thymocyte phenotype being associated with a better prognosis.

Cytogenetic and molecular genetic features

Approaching three-quarters of patients have an abnormal karyotype [187]. Cytogenetic

abnormalities may be non-specific (e.g. 6q− or 9p−), but in about a quarter of cases there is involvement of the TCR loci, particularly the TRA and TRD loci at 14q11.2 and the TRB locus at 7q34 but occasionally the TRG locus at 7p14.1. A complex karyotype, which is infrequent, is prognostically adverse [187]. Details of genetically defined categories are shown in Table 4.1 [16,184,189–207] and representative abnormalities are discussed in more detail below.

In addition to cytogenetic abnormalities, several deletions and point mutations are likely to be important in pathogenesis [185,209]; these occur in all the major cytogenetic subtypes. About 30% of patients have del(9)(p21.3) leading to homozygous loss of the tumour suppressor genes *CDKN2A* and *CDKN2B*; a similar number have a heterozygous deletion [209]. Heterozygous deletion of the tumour suppressor gene *CDKN1B* at 12p13 is found in about 12% of patients [209]. Activating mutations in *NOTCH1* are found in about 50% of patients [210], 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. *NOTCH1* and *FBXW7* mutations appear to correlate with a better prognosis.

Deletion or mutation of *WT1* is found in about 10% of cases [211]. Deletion or mutation of *LEF1* is present in about 15% of cases [212]. Deletions or gains that downregulate the tumour suppressor gene *TGFB1* are reported in 34% of patients, and deletions of gains that upregulate the PI3-AKT pathway in 29% of patients [209]. *PTEN* deletion or mutation is found in about 17% of cases and conveys resistance to NOTCH1 inhibitors [213]. *CASP8AP2* at 6q15 is deleted in 12% of patients, this correlating with a poor response to treatment [209]. *PHF6* deletions and inactivating mutations are found in about 16% of childhood and about 38% of adult cases [214]. These deletions and mutations of an X chromosome tumour suppressor gene are found almost exclusively in males [214]. Activating *JAK1* mutations are found in about 18% of adults with T-ALL and are associated with a poor prognosis [215]. Strong expression of *BAALC* is prognostically adverse [216]. Alterations of *BCL11B*, *MYB*, *RB1* and *PHF6* may also be implicated [4].

There is some correlation between genetic abnormalities and other features.

Cases expressing *LYL1* and those with a *KMT2A-MLLT1* fusion are usually CD4−CD8−CD34+ pro-T cells, those with *KMT2A-MLLT1* being TCR-γδ positive [217]. 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 [217].

About a fifth of patients have an IGH locus rearrangement in addition to the almost invariable rearrangement of TCR loci.

Early T-cell precursor ALL

This provisional WHO category of ALL represents about 12–16% of childhood cases and up to 10% of adult cases of T-ALL. Cases can be identified by their gene expression profile, which resembles that of a haemopoietic stem cell, or by their immunophenotype [4,186,218,219].

Clinical and haematological features

On average the WBC is lower than in other cases of T-ALL [219]. Prognosis was reported as very adverse [218]. In a UK trial in children and young adults this was not so [219], whereas in a US trial prognosis in adolescents and adults was adverse [220]. Adverse prognosis may be linked to CD33 expression rather than other features of the early T-cell phenotype [221].

Immunophenotype

The WHO definition of this category is that there is expression of CD3 and CD7; CD8 and CD1a are not expressed, and there is expression of one or more of CD34, CD117, HLA-DR, CD13, CD33, CD11b and CD65 [186]. CD3 expression may be membrane or cytoplasmic; CD5 expression is weak or absent [186,218]. In comparison with other T-ALL, there is less often expression of CD2, membrane CD3, CD4 and CD10, and more often expression of CD56 [222].

Table 4.1 Some genetic categories of T-lineage ALL. (Derived from references [16, 185, 190–208] and other sources.)

Cytogenetic abnormality	Mechanisms of leukaemogenesis	Approximate frequency
t(1;7)(p34;q34)	*LCK* gene at 1p34.3-35 dysregulated by proximity to the TRB 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 TRD locus at 14q11.2 or the TRB locus at 7q35	<1%
cryptic *TAL*[d]	*STIL-TAL1* fusion resulting from a cryptic deletion at 1p33	20–30% [190,191]
t(4;11)(q21;p15)	*NUP98-RAP1GDS4* fusion	5% [192]
cryptic t(5;14)(q35.1;q32.2)	*TLX3* (*HOX11L2*)-*BCL11A* (*CTIP1*)	[193]
cryptic t(5;14)(q35.1;q32.2)	*NKX2-5* dysregulation by proximity to *BCL11B*	[194]
t(6;7)(q23;q32)	*MYB* dysregulated by proximity to TRB at 7q34	[195]
t(6;11)(q27;q23.3)	*KMT2A-AFDN* (*MLLT4-AF6*) fusion	<1%
t(7;7)(p15;q34) inv(7)(p15q34)	*HOXA* gene cluster at 7p15-p.14.2 dysregulated by proximity to TRB locus at 7q34	[196]
t(7;9)(q34;q34.3)	*NOTCH*, 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 TRB locus at 7q34	<1%
t(7;10)(q35;q24) t(10;14)(q24;q11.2)	*TLX1 (HOX11)* at 10q24 dysregulated by proximity to the TRB locus at 7q35 or the TRD locus at 14q11.2	5–7% of children, 14–30% of adults [16,193]
t(7;11)(q35;p13)	*LMO2* (*RBTN2*) at 11p13, a TF gene, is dysregulated by proximity to the TRB locus at 7q34	2–7%
t(7;19)(q35;p13)	*LYL1* gene, a TF gene at 19p13.2-13.1, is dysregulated by proximity to the TRB locus at 7q34	<1%
t(8;14)(q24.1;q11)	*MYC* at 8q24.1 is dysregulated by proximity to the TRA/TRD locus at 14q11.2	1–2%
cryptic del(9)(q34.11q34.13)	*SET-NUP214* fusion	[197]
t(9;12)(p24;p13)	*ETV6-JAK2*	Rare [197]
t(9;12)(q34;p13)	*ETV6-ABL1*	[199]
cryptic t(9;14)(q34;q32)	*EML1-ABL1*	[200]
t(9;22)(q34;q11.2)	*BCR-ABL1*, more often m-BCR	Rare [201]
9q34 genes on episomes	*NUP214-ABL1* fusion	[202]
t(10;11)(p12–22;q23.3)	*KMT2A-MLLT10* (*AF10*) fusion	<1% [203,204]
Numerous	Other *KMT2A* rearrangements	5–10%
t(10;11)(p13;q14)	*PICALM-MLLT10* (*AF10*) fusion	9%, all TCRγδ expressing, of which it is found in 25% [205]

Table 4.1 (Continued)

Cytogenetic abnormality	Mechanisms of leukaemogenesis	Approximate frequency
t(11;14)(p15;q11)	*LMO1* (*RBTN1*) at 11p15, a TF gene, is dysregulated, by proximity to the TRD locus at 14q11.2	
t(11;14)(p13;q11)	*LMO2* (*RBTN2*) at 11p13, a TF gene is dysregulated, by proximity to the TRD locus at 14q11	<1%
t(11;19)(q23.3;p13.3)	*MLL-MLLT1* (*ENL*) fusion	3% [204]
t(12;21)(q11.2;q22)	*OLIG2* at 21q22 dysregulated by proximity to TRA/TRD locus at 14q32	[206]
inv(14)(q11q32) t(14;14)(q11;q32)	*TCL1A (TCL1)* at 14q32.1 is dysregulated by proximity to the IGH locus at 14q32.3	
inv(14)(q11.2q32.31)	*BCL11B-TRD*	[207]
t(X;7)(q22;q34)	*IRS4* dysregulated by proximity to TRB	Rare [208]

m-BCR, minor breakpoint cluster region; TF, transcription factor.

Cytogenetic and molecular genetic features

There is no characteristic cytogenetic abnormality but del(13q) may be more common than in other cases of T-ALL [218] and translocations involving *KMT2A* are more common than in other T-ALL [219]. The gene expression profile is that of an early T-cell progenitor. Molecular lesions differ between adult and childhood cases. In children *FLT3* mutations are common. In a series of adult patients, *FLT3* was mutated in 24/68, *NOTCH1* in 10/68 and *DNMT3A* in 10/68 [223]; *FLT3* mutations were either internal tandem duplications or tyrosine kinase domain mutations. In a series of children and young adults, typical T-ALL-related abnormalities, *NOTCH1* and *FBXW7* mutations, *CDKN2A* and *CDKN2B* deletions and translocations, and rearrangements activating T-cell specific oncogenes *LMO1*, *LMO2*, *TLX1*, *TLX3* and *STIL* were less common than in other cases of T-ALL [219]. Genes that are often mutated in AML are more frequently mutated than in other T-ALL: *FLT3*, *NRAS*, *KRAS*, *DNMT3A*, *IDH1* and *IDH2* [186]. A mutation in *CSFR3* was found in one of three patients investigated [224]; this gene is also mutated in chronic neutrophilic leukaemia so this observation emphasizes the myeloid potential of the cell that is mutated in this type of ALL. Other genes that may be implicated include *RUNX1*, *IKZF1*, *ETV6*, *GATA3*, *EP300*, *NRAS*, *IL7R*, *JAK1*, *JAK3*, *NF1*, *PTPN11* and *SH2B3* [4]. In addition there may be loss-of-function mutations in epigenetic regulator genes *EZH1* (previously *PRC2*), *EZH2*, *SUZ12*, *EED*, *H3K27* and *SETD2* [4].

Phenotypic and 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 4.1 that have not been incorporated into the WHO classification but that nevertheless have definable characteristics and illustrate mechanisms of leukaemogenesis. Whether these subtypes are of clinical significance is not yet clear. Some of these entities will now be discussed.

T lymphoblastic leukaemia/lymphoma with t(10;14)(q24;q11.2); *TLX1* dysregulation

This category of ALL comprises about 5–7% of childhood T-lineage ALL and about 14–30% of adult cases [16,193].

Clinical and haematological features

The majority of cases have FAB L1 cytological features. Prognosis is relatively good [217].

Immunophenotype

The immunophenotype may be early thymocyte [217] or intermediate or common thymocyte with coexpression of CD10 in a quarter of patients [16].

Cytogenetic and molecular genetic features

The mechanism of leukaemogenesis is dysregulation of *TLX1* (also known as *HOX11*), a transcription factor gene, as a consequence of proximity to the TRA/TRD locus at 14q11.2. A similar mechanism of leukaemogenesis is operative in T-lineage ALL with t(7;10)(q34;q24) when the *TLX1* gene is dysregulated by proximity to the TRB locus at 7q34. 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 [225].

T lymphoblastic leukaemia/lymphoma with t(5;14)(q35.1;q32.2) (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 [226].

Cytogenetic and molecular genetic features

The breakpoint on chromosome 5 is in the region of *TLX3* (also known as *HOX11L2*), which is transcriptionally activated, probably by proximity to the transcription regulatory elements of *BCL11B* (*CTIP2*) at 14q32.2 [193]. 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; *STIL-TAL1*

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 [190]. 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 [191]. Results in a small series of patients suggested a high WBC and an adverse prognosis [227].

Immunophenotype

No specific immunophenotype has been recognized except that this subtype of T-lineage ALL occurs preferentially among cases expressing TCRαβ and CD2, whereas CD10 is not expressed [226].

Cytogenetic and molecular genetic features

The mechanism of leukaemogenesis is that a small deletion at 1p33 leads to the fusion of most of the sequences of a transcription factor gene on chromosome 1, *TAL1* (*SCL*), with the promoter of the upstream *STIL* (*SIL*) gene. 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 *STIL* gene and the *TAL1* gene vary. The *TAL1* gene can also be dysregulated by translocations (see Table 4.1) but this is much less common than dysregulation as a consequence of a microdeletion.

Submicroscopic deletion involving 1p33, *TAL*[d], can be detected by PCR or RT-PCR [190]. 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 *STIL*–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) [202]. 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* [41].

Natural killer cell lymphoblastic leukaemia/lymphoma

Natural killer cell lymphoblastic leukaemia/lymphoma is a rare neoplasm; in the 2016 revision of the WHO classification it is included as a provisional entity within the category of precursor lymphoid neoplasms [228]. 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 neoplasms of immature NK cells [229]. There is expression of markers shared with the T lineage, such as CD7 and CD2 and cytoplasmic CD3ε, 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 IL15; CD56 is not expressed [229].

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 11q23.3 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 11q23.3 breakpoints and rearrangement of the *KMT2A* gene have included t(1;11)(p32;q23.3), t(4;11)(q21.3;q23.3), t(5;11)(q35;q23.3), t(11;16)(q23.3;p13) and t(11;19)(q23.3;p13) [230]. The great majority of cases are of B lineage but T-lineage ALL has also been reported associated with *KMT2A* rearrangement [230].

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) [231]. The latter category includes cases that were previously designated either biphenotypic or bilineal acute leukaemia.

Mixed phenotype 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 4.2 [231] together with the criteria for a diagnosis of acute undifferentiated leukaemia. Excluded from the category of MPAL are cases with defining genetic abnormalities of AML categories such as t(8;21), cases with rearrangement of *PDGFRA*, *PDGFRB*, *FGFR1* or with *PCM1-JAK2* and cases that meet the criteria for t-AML or myelodysplasia-associated AML [231]. MPAL is uncommon. In a series of 896 adults with acute leukaemia, 1.9% had MPAL [232]. B-myeloid cases are most frequent (in this series 11/17), followed by T-myeloid (5/17) and B-T (1/17) [232]. In a series of 4780 adult patients, the prevalence was 2.4%, of which 55% were B-myeloid, 32% T-myeloid

Table 4.2 WHO 2016 criteria for a diagnosis of mixed phenotype and undifferentiated acute leukaemia.

Lineage	Criteria
Myeloid*	Myeloperoxidase (any technique) *or* Expression of at least two of: diffuse non-specific esterase activity, CD11c, CD14, CD36, CD64 or lysozyme
T*	Cytoplasmic CD3 (but not expression only of CD3ζ) or surface membrane CD3
B*	Strong CD19 with strong expression of at least one of: CD79a†, cCD22 or CD10† *or* Weak CD19 with strong expression of at least two of: CD79a†, cCD22, CD10† ‡
Undifferentiated	No expression of cCD3, myeloperoxidase, cCD22 or cCD79a, absence of strong expression of CD19

c, cytoplasmic.
* If the case meets criteria for assignment to two or more lineages the case is categorized as mixed phenotype acute leukaemia.
† 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.
‡ PAX5 expression may also be taken as evidence of B lineage.
Adapted from Borowitz *et al*. 2017 [231].

and 12% B-T with one case of trilineage acute leukaemia [233]. Clinical features include a high prevalence of hyperleucocytosis and CNS disease [234]. Morphological features (Fig. 4.15) are most often 'lymphoid' but can be 'undifferentiated' or myeloid or there may be a dimorphic blast population [234].

About a third of MPAL cases are Ph positive [235,236] but the frequency is much lower in children, 4.4% in one series [234]. The second commonest group is associated with a variety of abnormalities with an 11q23.3 breakpoint and *KMT2A* rearrangement, particularly t(4;11) and del(11)(q23). In a series of children, 4 of 33 patients with MPAL (12%) who had successful cytogenetic analysis had *KMT2A* rearrangement [237], and in another childhood series *KMT2A* rearrangement was found in 10.9% [234]. A significant proportion of the rare leukaemias associated with t(10;11)(p12.3;q14.2), involving the *MLLT10* and *PICALM* genes, are of mixed phenotype, particularly myeloid and T lineage but occasionally myeloid, T and B lineage [238]. Other cytogenetic abnormalities found include trisomy 8 (13.5% in a series of children) [234], a complex karyotype (11.5% in the same series) [234], monosomy 7 [233] and polysomy 21 [233].

Lineage switch, for example from AML to T-ALL [239] or from acute erythroleukaemia to precursor B-ALL [240], represents a form of MPAL. It is necessary to demonstrate the same or an evolved cytogenetic abnormality to confirm this diagnosis.

Mixed phenotype acute leukaemias are generally considered to have a poor prognosis, possibly but probably not only, because of the frequent association with an adverse karyotype. However, in one study in children, prognosis although worse than that of ALL was similar to that of AML [237]. In another childhood series the response rate was worse than that of ALL but comparable with AML, while the 3-year event-free survival was intermediate between that of ALL and that of AML [234].

Mixed phenotype acute leukaemia with t(9;22)(q34.1;q11.2) and *BCR-ABL1*

t(9;22)(q34.1;q11.2) is associated with a variety of types of biphenotypic leukaemia, particularly with cases showing evidence of myeloid

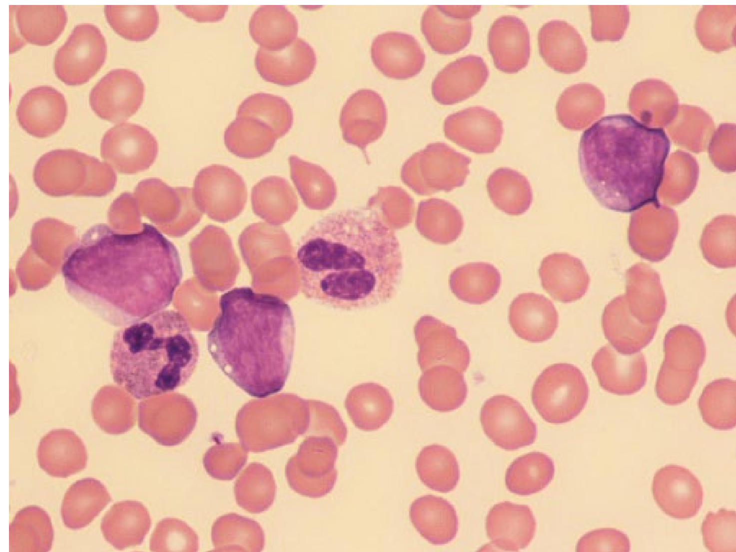


Fig. 4.15 PB film in mixed phenotype (B-myeloid) acute leukaemia showing three blast cells with small cytoplasmic vacuoles but otherwise no specific cytological features. Immunophenotyping showed expression of (i) CD34 and terminal deoxynucleotidyl transferase; (ii) CD10, CD19, CD79a and cytoplasmic CD22; and (iii) CD15, CD33, myeloperoxidase and lysozyme.

and B-lymphoid differentiation but also with occasional cases with B- and T-lymphoid or myeloid, B-, and T-lymphoid differentiation [236]. 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. 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 myeloid leukaemia. There are often additional cytogenetic abnormalities, sometimes with a complex karyotype.

Mixed phenotype acute leukaemia with t(4;11)(q21.3;q23.3) and *KMT2A-AFF1* or other rearrangement of 11q23.3/*KMT2A*

t(4;11)(q21.3;q23.3) is associated with ALL with an early-B-precursor phenotype, with acute monoblastic leukaemia and with MPAL. In *KMT2A*-rearranged 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 11q23.3/*KMT2A* breakpoint have also been associated with MPAL. Other partner chromosomes have included 9 (*MLLT3*), 10 (*MLLT10*), 17 and 19 (*MLLT1*) [236,237,241–244].

In addition to presentation with MPAL, rearrangements of *KMT2A* can be associated with rapid lineage switch during treatment, most often from B-lineage ALL to AML, but sometimes the reverse [244]. The AML is monocytic/monoblastic or, less often, myelomonocytic. Both lymphoid to myeloid and mixed phenotype myeloid/lymphoid (with minor lymphoid component) to lymphoid switch have been observed in association with t(4;11) [244]. Lymphoid to myeloid switch has been observed with t(9;11) and t(11;19) [244].

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

This rare type of leukaemia has no specific distinguishing characteristics. Recurrent chromosomal abnormalities that have been described

include cryptic del(1)(p32) with *STIL-TAL*, t(2;5)(p13;p13–15.3), trisomy 4, t(4;12)(q12–21;p13), del(5q), monosomy 5, del(6p), del(6q), monosomy 7, del(7p), del(7q), structural abnormalities of chromosome 7, trisomy and polysomy 8, t(10;11)(p12.2;q14.2) with *PICALM-MLLT10*, abnormalities of 12p11.2, t(12;22)(p13;q12), polysomy 21, abnormalities of the X chromosome, and aneuploidy including hyperdiploidy and near tetraploidy [231,245,246]. A small number of adults have been described with L3 ALL morphology, typical B-lineage markers, and both cytochemical and antigenic positivity of blast cells for MPO [247]; four of five had complex cytogenetic abnormalities and all responded to chemotherapy appropriate for ALL [247].

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. Other recurrent chromosomal abnormalities that have been observed include t(2;5)(p13;p13–15.3), t(3;11)(q13;p15), trisomy 4, monosomy 5, del(5q), del(6q), cryptic t(7;12)(q36;p13) with *KRT86-ETV6* (previously *MNX-ETV6*), t(8;12)(q13;p13) with *ETV6-NCOA2*, t(10;11)(p12.2;q14.2) with *PICALM-MLLT10*, rearrangement of 14q32, i(17)(q10), trisomy 19, polysomy 21, abnormal X chromosome and hyperdiploidy [246].

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. Recurrent cytogenetic abnormalities that have been observed in B/T/myeloid acute leukaemia include trisomy 4, del(6q), hyperdiploidy and complex karyotypes [247].

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 4.2. Recurrent cytogenetic abnormalities that have been observed include del(5q), trisomy 12, trisomy 13 and del(20q) [246].

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.

Conclusions

As for AML, the classification of ALL and MPAL has become increasingly complex, and this trend is likely to continue as new molecular categories are discovered to have prognostic and therapeutic importance.

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5

The Myelodysplastic Syndromes and the Myelodysplastic/Myeloproliferative Neoplasms

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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, 2008 and 2016 [6–8].

Overall the incidence of MDS is about three times that of AML [9] and rises exponentially with age. Most published estimates fall between 3 and 12/100 000/year, with higher estimates more likely to be correct [9]. 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 [9]. The incidence in men is about twice that in women [9,10] and is higher in White Americans than Black or Asian Americans [10].

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

Table 5.1 Features of some inherited conditions predisposing to myelodysplastic syndromes [14].

Condition	Gene/s involved	Haematological characteristics	Common associated cytogenetic abnormalities
Fanconi anaemia	Multiple	Bone marrow failure	Duplication of 1q and 3q, del(7q), monosomy 7, del(11q)
Dyskeratosis congenita	Multiple	Bone marrow failure	
Shwachman–Diamond syndrome	*SBDS*	Neutropenia, sometimes anaemia or thrombocytopenia	iso(7)(q10), del(20)(q11)
Diamond–Blackfan anaemia	Multiple ribosomal genes	Red cell aplasia	
Congenital amegakaryocytic thrombocytopenia	*MPL*	Thrombocytopenia	Monosomy 7, trisomy 8
Familial platelet disorder with propensity to acute myeloid leukaemia	*RUNX1*	Thrombocytopenia	Trisomy 21
CEBPA-associated familial MDS/AML	*CEBPA*		
GATA2 haploinsufficiency (Mono/MAC syndrome)	*GATA2*		Monosomy 7, trisomy 8
SRP72-associated familial aplastic anaemia/MDS	*SRP72*	Familial aplastic anaemia	

may be principally manifest in one lineage (the granulocytic/monocytic lineage, the erythroid lineage or, less often, the megakaryocyte lineage) but commonly dysplasia is bilineage or trilineage. 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 topoisomerase 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) [11]. An association with alemtuzumab therapy has also been suspected [12]. There are also genetic disorders that predispose to MDS; these include Diamond–Blackfan anaemia, Fanconi anaemia, Rothmund–Thomson syndrome [13], dyskeratosis congenita, Shwachman–Diamond syndrome and Bloom syndrome (Table 5.1) [14]. 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 [15] 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 5.2 [16–29]. The usefulness of such features in diagnosis varies. Some abnormalities, such as an acquired Pelger–Huët anomaly (Fig. 5.1) and micromegakaryocytes (Fig. 5.2), are highly characteristic and almost pathognomonic of MDS; one or other of these abnormalities occurs in a high percentage of patients [30]. Agranular neutrophils (Fig. 5.3) are also highly specific but are present in a smaller proportion of 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. 5.26). Other abnormalities such as macrocytosis, elliptocytosis, red cell fragmentation (Fig. 5.4) or the presence of other poikilocytes, monocytosis, neutropenia, heavy granules in neutrophils or precursors (Fig. 5.5), binucleate or other apparently tetraploid neutrophils (Fig. 5.6) and cytoplasmic abnormalities in megakaryocytes [31] 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 304), immunophenotyping, bone marrow histology (see page 306), bone marrow culture (see page 316) and cytogenetic or molecular genetic analysis to demonstrate acquired clonal abnormalities (see pages 310–315).

Myelodysplastic syndrome needs to be distinguished from the myeloproliferative neoplasms (MPN), among which are included polycythaemia vera, essential thrombocythaemia, primary myelofibrosis and chronic myeloid leukaemia. 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 the MDS/MPN category in acknowledgement of its overlapping features [6,32]. 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 in the WHO classification to the MDS/MPN category [32], with the exception of cases that meet the criteria for MDS associated with isolated del(5q), which continue to be classified as MDS. 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 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 myeloid 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. 5.7.

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

Table 5.2 Haematological features that may occur in the myelodysplastic syndromes (MDS) [16–29].

Peripheral blood	Bone marrow
Erythropoiesis	
Anaemia and red cell dysplasia Normocytic normochromic (common) Macrocytic (common) Microcytic (uncommon)* Dimorphic blood film Anisocytosis, anisochromasia Poikilocytosis (which may include ovalocytes, elliptocytes† [16–18], schistocytes [16,19], teardrop poikilocytes, stomatocytes, acanthocytes [20] 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) [21]	Hyperplasia (common) Hypoplasia (uncommon) including red cell aplasia Megaloblastic erythropoiesis Macronormoblastic erythropoiesis Sideroblastic erythropoiesis 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
Granulopoiesis	
Neutropenia (common) Neutrophilia (uncommon) Acquired Pelger–Huët anomaly Neutrophils with hypersegmented nuclei, increased nuclear projections, ring nuclei or nuclei of bizarre shape, increased chromatin clumping, detached nuclear fragments‡, increased apoptotic forms Agranular and hypogranular neutrophils Hypergranular neutrophils or giant granules (uncommon) Persistence of cytoplasmic basophilia in mature neutrophils or presence of Döhle bodies Macropolycytes and binucleated neutrophils Monocytosis, abnormal monocytes Presence of promonocytes Blast cells, with or without Auer rods Eosinophilia (uncommon) Hypogranular eosinophils and eosinophils with ring-shaped nuclei or non-lobulated nuclei Basophilia (uncommon)	Granulocytic hyperplasia Granulocytic hypoplasia Increased blast cells, with or without Auer rods or with giant (pseudo-Chédiak–Higashi) granules Hypogranular or hypergranular promyelocytes Hypogranular myelocytes Increased chromatin clumping in myeloid precursors Increased monocytes and promonocytes Cytoplasmic vacuolation Lack of mature neutrophils Morphologically abnormal neutrophils Increased§ or dysplastic eosinophils [22] including eosinophils with intranuclear Charcot– Leyden crystals [23] (uncommon) Increased basophils¶ [22] Increased mast cells Atypical mast cells [24]
Thrombopoiesis	
Thrombocytopenia (common) Thrombocytosis (uncommon) Giant platelets Hypogranular or agranular platelets Platelets with giant granules Micromegakaryocytes Cyclical thrombocytopenia (rare) [25]	Reduction of megakaryocytes Increase of megakaryocytes Mononuclear or binuclear micromegakaryocytes Megakaryocytes with non-lobated or hypolobulated nuclei Multinucleated megakaryocytes Megakaryocytes with botryoid nuclei Hypogranular megakaryocytes

* Microcytic anaemia in MDS may be consequent on acquired haemoglobin H disease [26] or, rarely, acquired α [27] or β thalassaemia trait [28]; acquired α thalassaemia/haemoglobin H disease results from a mutation in the *ATRX* gene, which encodes a transcriptional cofactor [29]; microcytosis 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 [17] and to del(20q) [18].

‡ 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 [22].

¶ In one series of patients, bone marrow basophils were above 1% in 11.8% of patients [22]

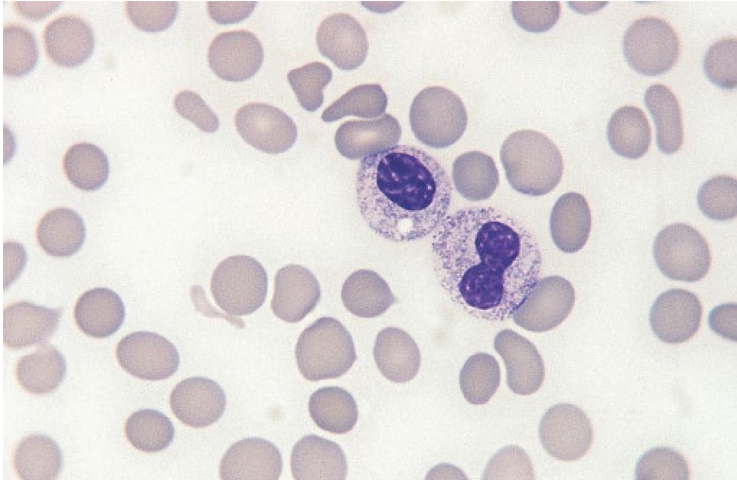


Fig. 5.1 Peripheral blood (PB) film of a patient with therapy-related myelodysplastic syndrome (MDS) showing the acquired Pelger–Huët anomaly; also apparent are anisocytosis, poikilocytosis and severe thrombocytopenia. The bone marrow (BM) showed trilineage myelodysplasia. May–Grünwald–Giemsa (MGG) ×100.

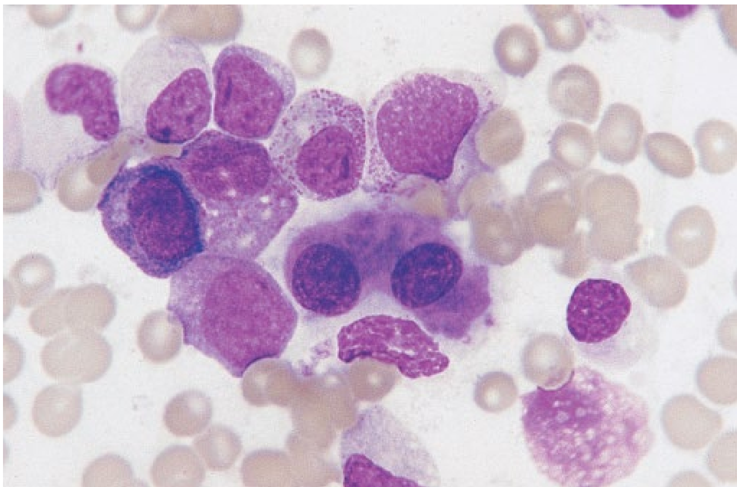


Fig. 5.2 A binucleated micromegakaryocyte in the BM of a patient with refractory cytopenia with multilineage dysplasia (RCMD)/MDS with multilineage dysplasia (MDS-MLD); there was also granulocytic hyperplasia, hypogranularity of the neutrophil series and severe erythroid hypoplasia. MGG ×100.

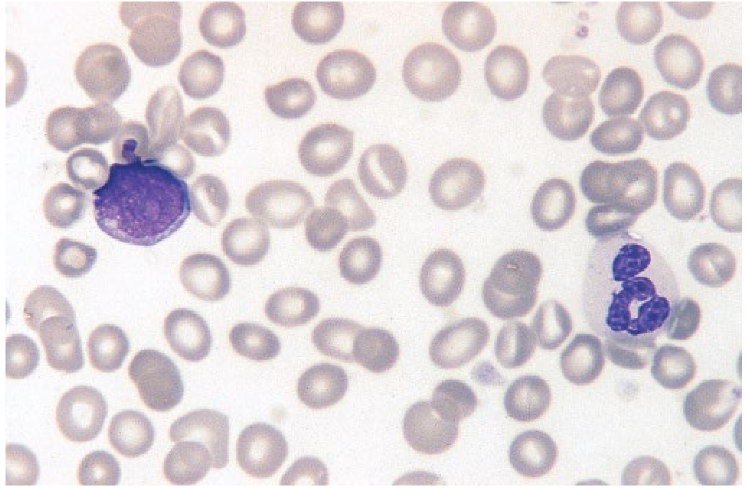


Fig. 5.3 Agranular neutrophil in the PB of a patient with refractory anaemia with excess of blasts (RAEB)/MDS with excess blasts (MDS-EB). A myeloblast is present, some red cells are stomatocytic and platelet numbers are markedly reduced. MGG ×100.

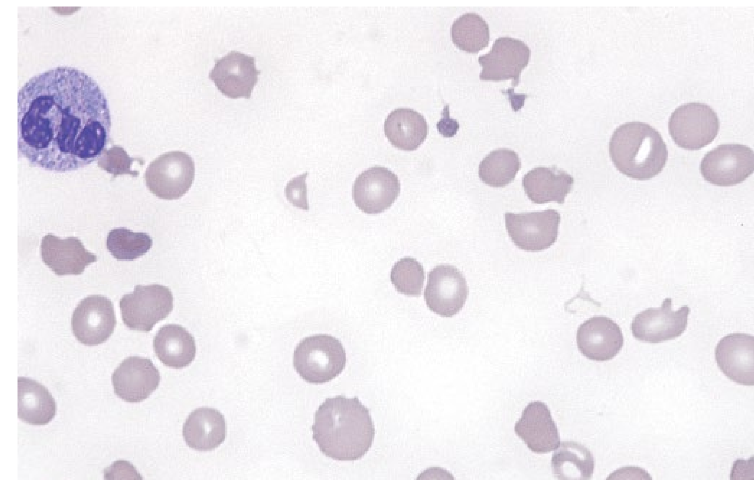


Fig. 5.4 PB film of a patient with RCMD/MDS-MLD showing marked poikilocytosis including the presence of several fragments and one stomatocyte. There is also anisocytosis with the presence of several macrocytes. Platelet numbers are greatly reduced and BM examination showed megakaryocytic as well as erythroid dysplasia. MGG ×100.

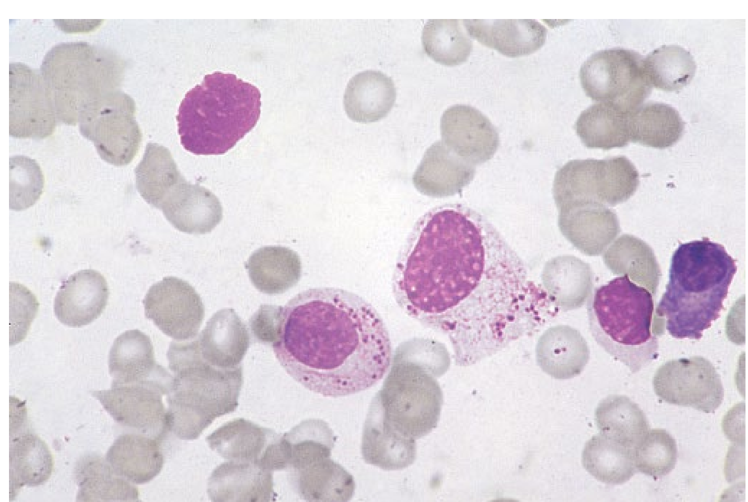


Fig. 5.5 Granulocyte precursors with abnormally heavy granules from a patient with MDS. MGG ×100. (With thanks to the late Dr David Swirsky.)

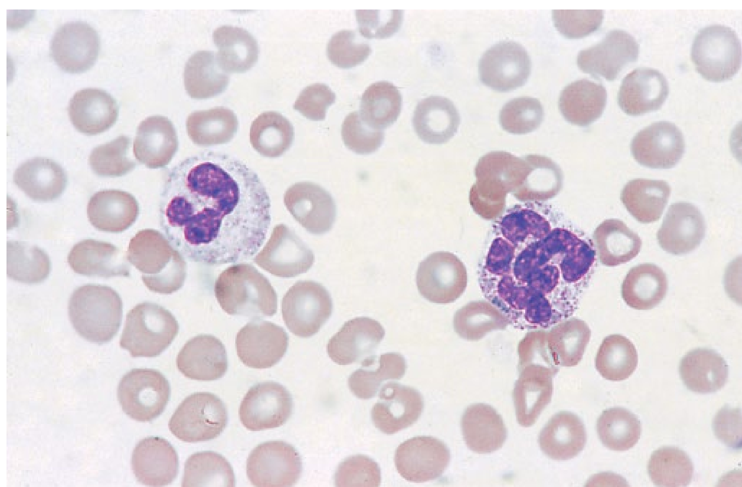


Fig. 5.6 PB film showing a normal neutrophil and a macropolycyte, probably a tetraploid cell, in a patient with MDS. MGG ×100.

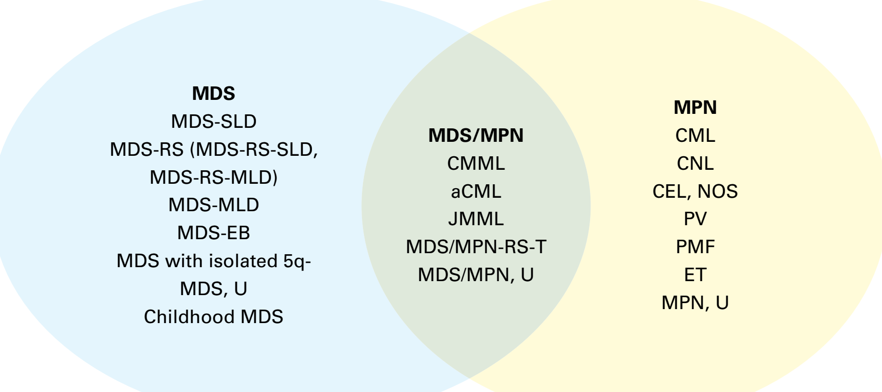


Fig. 5.7 The relationship between the myelodysplastic syndromes, the myeloproliferative neoplasms and the myelodysplastic/myeloproliferative neoplasms in the World Health Organization (WHO) classification using the revised 2016 terminology, *de novo* cases only. aCML, atypical chronic myeloid leukaemia; CEL, NOS, chronic eosinophilic leukaemia, not otherwise specified; CML, chronic myeloid leukaemia; CMML, chronic myelomonocytic leukaemia; CNL, chronic neutrophilic leukaemia; ET, essential thrombocythaemia; JMML, juvenile myelomonocytic leukaemia; MDS, myelodysplastic syndrome; MDS-EB, MDS with excess blasts; MDS-MLD, MDS with multilineage dysplasia; MDS/MPN, myelodysplastic/myeloproliferative neoplasm; MDS/MPN-RS-T, MDS/MPN with ring sideroblasts and thrombocytosis; MDS/MPN, U, MDS/MPN, unclassifiable; MDS-RS, MDS with ring sideroblasts; MDS-RS-MLD, MDS with ring sideroblasts and multilineage dysplasia; MDS-RS-SLD, MDS with ring sideroblasts and single lineage dysplasia; MDS-SLD, MDS with single lineage dysplasia; MDS, U, MDS, unclassifiable; MPN, myeloproliferative neoplasm; MPN, U, MPN, unclassifiable; PMF, primary myelofibrosis; PV, polycythaemia vera.

generalizations are suggested by studies of glucose-6-phosphate dehydrogenase (G6PD) alloenzymes [33], by cytogenetic analysis [34] and by investigation of *RAS* gene mutations and active and inactive alleles of X chromosome genes [35]. Lineages that appear morphologically normal may be part of the abnormal clone, and in some cases the T lymphocytes [34], both T and B lymphocytes [33,35] or B lymphocytes and natural killer cells [36] 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 or molecular abnormalities and showing itself in progressive marrow failure, an increase in the number of blast cells or the development of overt acute leukaemia. Supervening acute leukaemia is usually myeloid but occasionally lymphoblastic. In rare cases, cytogenetic analysis has proven that the lymphoblasts arise from the myelodysplastic clone [37].

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 5.3 [4,5]. Median survivals and the likelihood of progression to AML in different FAB categories are given in the previous edition of this book [38]. 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

Table 5.3 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.

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, 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 cases with unilineage and cases with multilineage dysplasia, these were assigned to separate groups. A distinct, good prognosis group was identified associated with isolated del(5q) (specifically an interstitial deletion of part of the long arm of chromosome 5), which became a separate WHO category.

Other changes related to the FAB categories of RAEB and RAEB-T (refractory anaemia with excess of blasts in transformation). In the WHO classification, RAEB was stratified into two prognostic groups, RAEB-1 and RAEB-2. The FAB category of RAEB-T was abolished in the WHO classification, cases having a bone marrow blast count of 20% or more being reclassified as AML, while cases with Auer rods were classified as RAEB-2. Another group of patients who would have met the FAB numerical criteria for MDS were reclassified as AML, regardless of the blast count, on the basis of the presence of either t(8;21)(q22;q22.1) or inv(16)(p13.1q22)/t(16;16)(p13.1;q22). The reason for this change was 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. The WHO acknowledged difficulties in classification by creation of a category designated 'MDS, unclassifiable'.

The WHO classification permits 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 (see below).

The WHO classification requires 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 [39]. Nevertheless, the WHO classification and all prognostic scoring systems are based on the number of blast cells counted in blood and bone marrow films.

The 2008 WHO classification [7], is now widely used and both it and it 2016 revision have replaced the FAB classification, which will therefore not be discussed in detail. Readers who require further information are referred to the previous two editions of this book [38,40] 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, or CAE) may show mature neutrophils with negative reactions [41,42] 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 [43]. The neutrophil alkaline phosphatase (NAP) 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 [43].

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 [44]. 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 [41]. 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 page 347). A minority of cases of MDS have acquired haemoglobin H disease [26]; they can be identified with a cytochemical stain using new methylene blue or brilliant cresyl blue to detect haemoglobin H inclusions (as well as by haemoglobin electrophoresis or high-performance liquid chromatography).

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. 5.8).

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 [45–52]. A basic panel of 20 antibodies for four-colour flow cytometry has been proposed by the European LeukemiaNet [51]. Abnormalities demonstrated in the granulocytic lineage may include detection of hypogranular neutrophils (identified by reduced side scatter – SSC – of light), a population of cells in the 'blast window' (identified on a CD45/SSC plot), abnormal antigen expression by blast cells (e.g. reduced or even absent CD45 expression or increased CD45 expression; lack of expression of human leucocyte antigen DR (HLA-DR), CD13 or CD33; reduced CD38 expression; inappropriate expression of CD2, CD5, CD7, CD10, CD11b, CD16, CD19 or CD56); expression of CD11b or CD15 on immature cells of granulocyte lineage [52]; and detection of abnormal antigen expression by maturing cells, either granulocytes or monocytes (reduced expression of CD10, CD11b, CD14, CD16 or CD33,

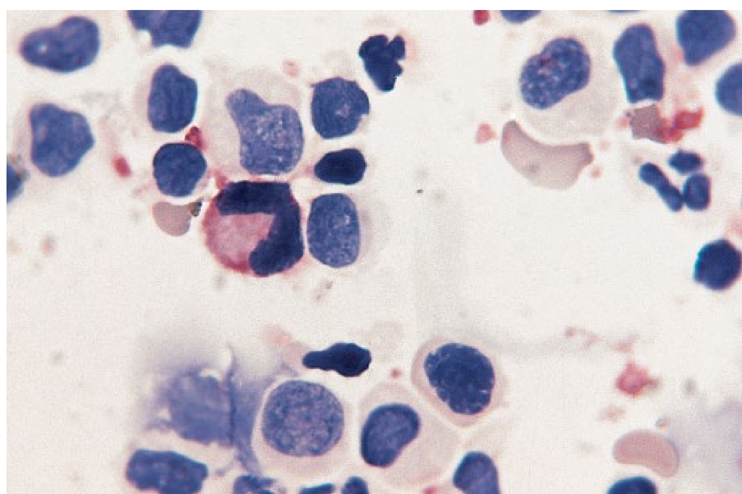


Fig. 5.8 Immunocytochemistry of a BM film from a patient with refractory anaemia with excess of blasts in transformation (RAEB-T) in the French–American–British (FAB) classification, classified as acute myeloid leukaemia in the WHO classification, showing several platelets and a micromegakaryocyte, which have given positive reactions with a monoclonal antibody directed at platelet glycoprotein Ib (CD42b). Alkaline phosphatase–anti-alkaline phosphatase (APAAP) technique ×100.

retention of CD34, HLA-DR, terminal deoxynucleotidyl transferase or CD117 expression on maturing cells, or expression of non-myeloid antigens such as CD5, CD7 or CD56) [45,46,49,50,53]. It should be noted that CD7 can be expressed on a small proportion of normal early myeloblasts [51]. Abnormalities detected in erythroid cells include reduced CD71 expression and asynchronous expression of CD71 or glycophorin A in comparison with CD45 [45] and downregulation of CD36 [50]. A combination of underexpression of CD71 and overexpression of CD105 was found to have 75% sensitivity and 92% specificity for distinguishing MDS from non-clonal cytopenia [54]. 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 [46] and has been found to add extra prognostic information to the low-risk group of the International Prognostic Scoring System, Revised (IPSS-R) prognostic scoring system [55]. 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 SSC (reduced) [53]. Expression of CD7 and CD56 on myeloid blasts has been found to be an independent adverse prognostic indicator [56]. Immunophenotyping of red cells or neutrophils may demonstrate the presence of a paroxysmal nocturnal haemoglobinuria (PNH) clone, which correlates with a better prognosis [57].

Flow cytometry shows the proportion of B-cell progenitors (haematogones) to be decreased in MDS [51] but there is also a decrease in old age, and there is some overlap with values in cytopenic patients without MDS.

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) [49], following growth factors (aberrant CD56 expression) [51], in sepsis (aberrant expression of CD64 on neutrophils and aberrant CD56 expression on neutrophils and monocytes) [49,51], in systemic lupus erythematosus (reduced monocyte expression of CD14 and HLA-DR) [58], in HIV infection (neutrophils showing reduced SSC, CD16 and CD11b) and no doubt in other non-neoplastic conditions characterized by dysplasia.

There appears to be little relationship between immunophenotype and WHO categories of MDS.

Immunophenotyping can also be carried out by immunohistochemistry in trephine biopsy sections (see below).

Bone marrow trephine biopsy

Attempting to identify specific WHO subtypes from trephine biopsy sections is not advised since diagnostic criteria depend on precise quantification of cell types and cytological abnormalities. However, a core biopsy often gives extra information not provided by an aspirate [59–63] so that the two investigations are complementary. Cellularity can be more reliably assessed on sections and any increase in 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. 5.9). They may show an excess of proerythroblasts (Fig. 5.10) or have all precursors at the same stage of development [59]. 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 5.11 and 5.12). This phenomenon has been designated 'abnormal localization of immature precursors' (ALIP) [59]. ALIPs can be diagnostically important in confirming MDS rather than a secondary anaemia. Abnormal megakaryocytes (binucleated, multinucleated or hypolobated) are readily assessed from biopsy sections (Fig. 5.13). Megakaryocytes may be clustered or found in a paratrabecular position (Fig. 5.14).

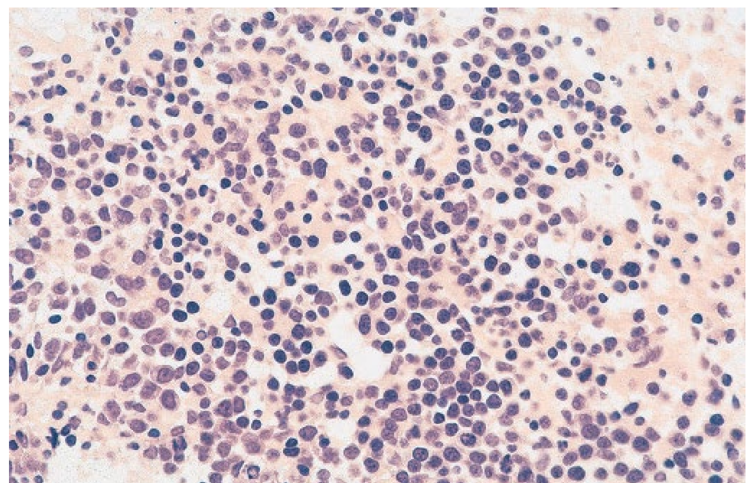
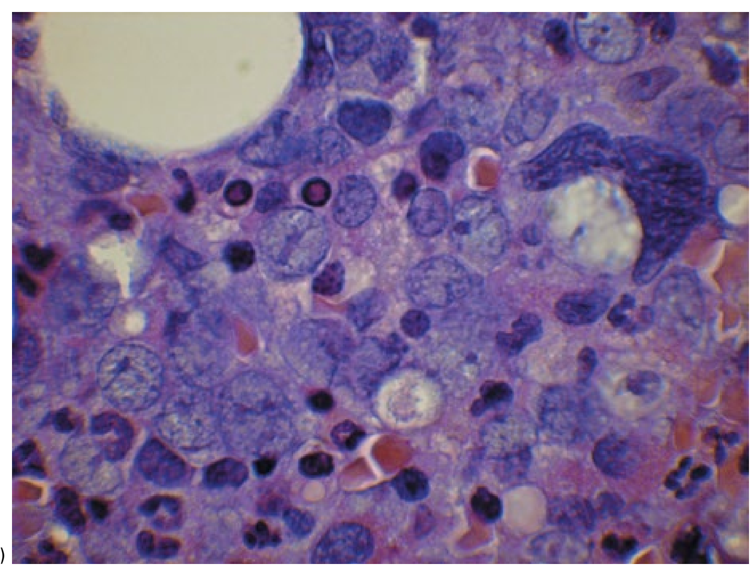


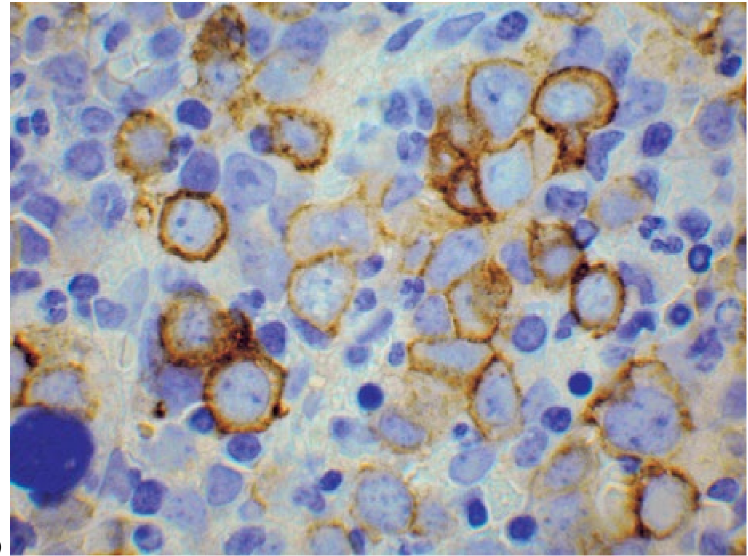
Fig. 5.9 Histological section of a trephine biopsy specimen from a patient with RAEB showing a large ill-formed erythroid island. Haematoxylin and eosin (H&E) ×40.



(a)

(b)

Fig. 5.10 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;



(c)

Fig. 5.10 (Continued)
(c) immunoperoxidase for CD117 (which can be expressed by proerythroblasts as well as by early cells of granulocyte lineage) ×100. (With thanks to the late Dr Alan Mills.)

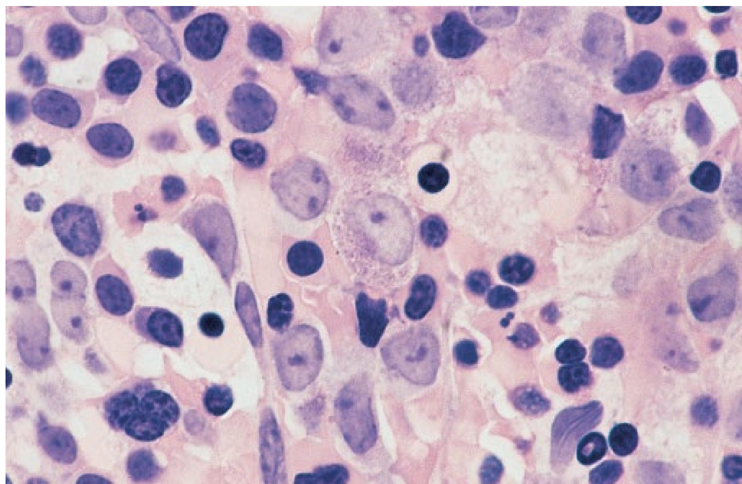


Fig. 5.11 Histological section of a trephine biopsy specimen from a patient with RAEB-T showing erythroblasts, some of which are dysplastic, and a collection of blasts and promyelocytes that are not adjacent to bone – 'abnormal localization of immature precursors' (ALIP). H&E ×100.

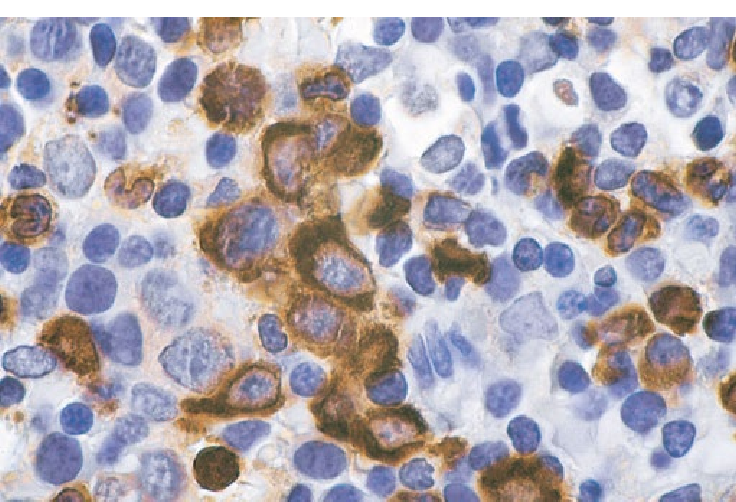


Fig. 5.12 Histological section of a trephine biopsy specimen from a patient with RARS/MDS-RS-SLD showing an ALIP demonstrated by immunohistochemistry. Immunoperoxidase ×100.

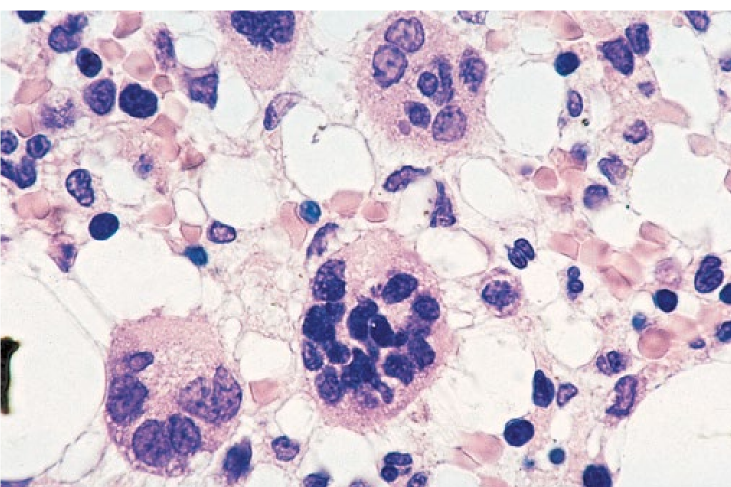


Fig. 5.13 Histological section of a trephine biopsy specimen from a patient with RAEB/MDS-EB showing dysplastic megakaryocytes and general disorganization of BM architecture. H&E ×100.

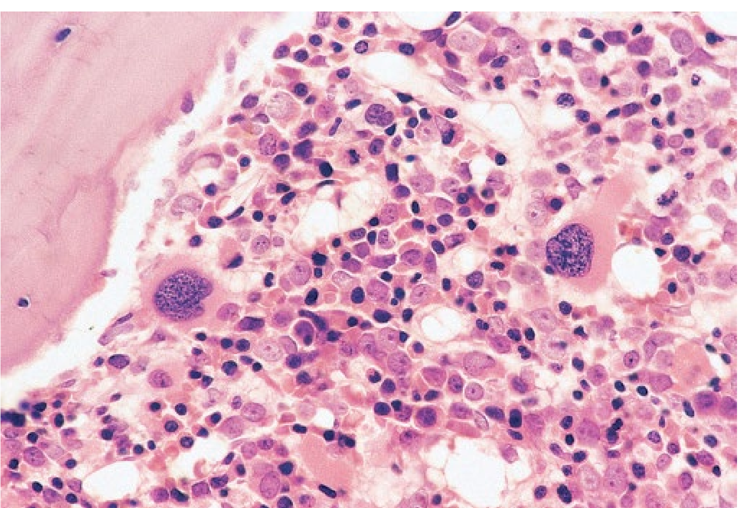


Fig. 5.14 Histological section of a trephine biopsy specimen from a patient with MDS showing abnormal megakaryocyte topography; a megakaryocyte is abnormally sited, adjacent to the bony trabecula. H&E ×40. (With thanks to Dr Simon Davies.)

Dysgranulopoiesis and dyserythropoiesis may be detectable but they are more readily apparent in a bone marrow film. Apoptosis is increased [64]. Nodules of immunophenotypically abnormal monocytes (or plasmacytoid dendritic cells) are sometimes present [65]. 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.

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 also helpful in distinguishing hypocellular MDS, which may have increased reticulin and foci of blasts, from aplastic anaemia, which does not show these features.

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. Expression of granulocyte antigens can confirm the nature of ALIPs (see Fig. 5.12). Polyclonal antibodies directed at von Willebrand factor or monoclonal antibodies recognizing epitopes on platelet antigens, such as CD42a, CD42b or CD61, highlight megakaryocytes and facilitate the recognition of micromegakaryocytes. 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 [65]. Reactions with some antibodies give prognostic information [66]. 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 higher grade MDS 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.

Cytogenetic features

Various clonal cytogenetic abnormalities have been described in association with MDS [67–77] (Table 5.4), 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. 5.15) and trisomy 8. It should be noted that monosomy 5 usually results from an unbalanced translocation with retention of 5p sequences. Some abnormalities are particularly characteristic of t-MDS (see Table 5.3). With the exception of del(5q), cytogenetic abnormalities are probably mainly secondary events. MDS is commonly associated with loss of chromosomal material, either through monosomy, deletion or unbalanced translocation. There can also be chromosomal rearrangements leading to both loss and gain of chromosomal material, for example +der(1)t(1;16)(p11;p11.1) in which there is gain of 1q and loss of 16q. Balanced translocations are less common.

Clonal abnormalities and, in particular, complex karyotypic abnormalities are more common in higher grade MDS. In addition, adverse cytogenetic abnormalities are more common when there is multilineage rather than unilineage dysplasia [78,79]. Cytogenetic analysis is essential for recognition of the '5q– syndrome' as an entity (see below), and certain abnormalities can also permit a case to be recognized as MDS when cytological evidence is insufficient.

Certain cytogenetic abnormalities are associated with particular clinical or morphological features. Monosomy 7 is more often found when there is pancytopenia, a hypocellular bone marrow and trilineage myelodysplasia, and is associated with a relatively poor prognosis. Hypocellular MDS has also shown an association with trisomy 6, trisomy 8, del(7q) and del(5q). Del(11q) has been associated with increased ring sideroblasts. Marked elliptocytosis has been associated with del(20q), this cytogenetic abnormality being observed in six of nine studied cases [18]. Isolated del(20q) is also significantly associated with lower blast cell counts [80] and thrombocytopenia [80,81].

Abnormalities of 3q21.3 and 3q26.2 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 3q21.3q26.2 abnormalities will meet the diagnostic criteria for MDS while others have MDS/MPN or AML. Those who meet the diagnostic criteria for MDS have a high probability of progression to AML and a poor prognosis [82].

Table 5.4 Cytogenetic abnormalities associated with the myelodysplastic syndromes.

	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		11q23.3 rearrangements including: t(2;11)(p21;q23.3) t(9;11)(p21;q23.3)* t(11;16)(q23.3;p13.3)* del(11)(q23.3) i(17q) 21q22.1 rearrangements including: t(3;21)(q26.2;q22.1)*
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) Other 3q21.3 and/or 3q26.2 rearrangements: t(1;3)(p26;q21.3)* inv(3)(q21.3q26.2)* t(3;3)(q21.3;q26.2)* del(3)(q21.3) ins(3)(q26.2;q21.3q26.2) t(3;4)(q26.2;q21) t(3;5)(q21.3;q31)* t(3;8)(q26.2;q24) t(3;12)(q26.2;p13) t(3;19)(q21.3;p13) t(3;5)(q25;q34) t(6;9)(p23;q34.1) 11q23.3 rearrangements less often associated with MDS including: t(1;11)(p32;q23.3)* t(3;11)(p21;q23.3) t(11;17)(q23.3;q25) t(11;19)(q23.3;q13.1)* t(11;21)(q23.3;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 [69].
§ 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.
Derived from references 67–77 and other sources.

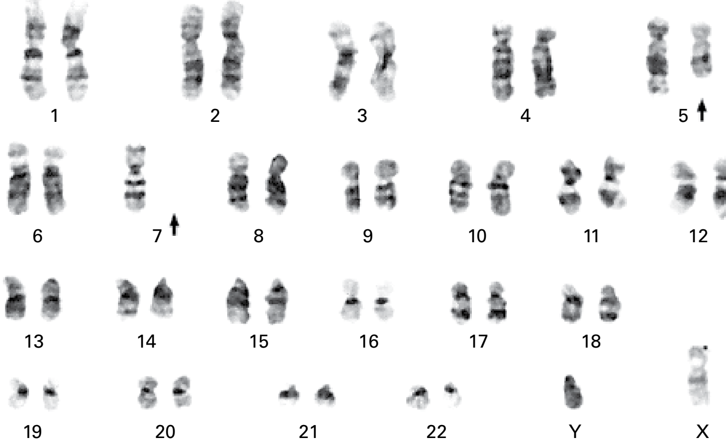


Fig. 5.15 Karyogram of a male patient with MDS showing a partial deletion of the long arm of chromosome 5 and monosomy 7. The karyotype was 45,XY,del(5)(q21q33),–7. (With thanks to Professor Lorna Secker-Walker.)

Deletion of the short arm of chromosome 17, with formation of an isochromosome of the long arm, i(17q) (Fig. 5.16), 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. 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 [83–85]. The neutrophils may also be hypogranular and they may have ring nuclei [85]. There is usually trilineage myelodysplasia (including micromegakaryocytes [85]) and although the blast count is often less than 5% at presentation there is rapid progression to AML [86]. There may be myeloproliferative as well as myelodysplastic features, with most patients having leucocytosis and monocytosis and some having thrombocytosis [86], this leading to categorization of these cases as MDS/MPN. Immature granulocytes may be increased in the peripheral blood, some patients have eosinophilia and a smaller number have basophilia [86]. A trephine biopsy shows increased reticulin fibrosis [86]. 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

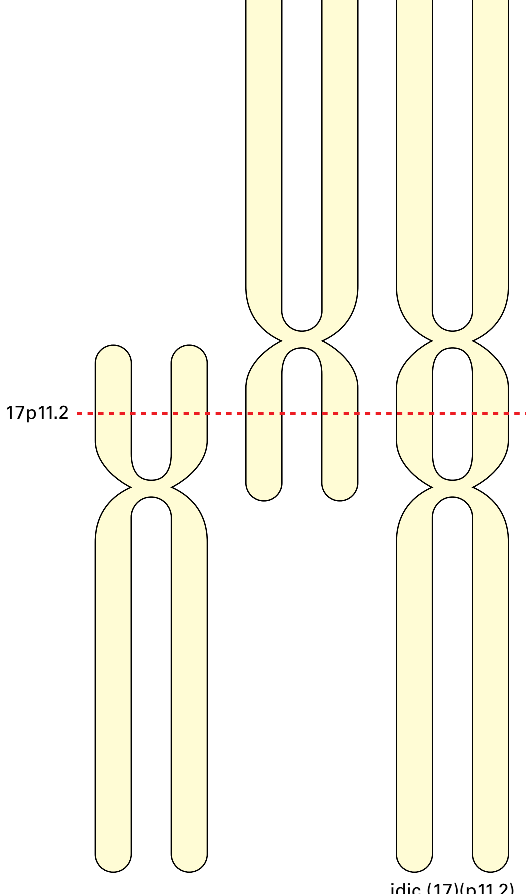


Fig. 5.16 Diagram showing the development of idic(17)(p11.2), the commonest form of i(17q).

17p including 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.

A 'monosomal karyotype', defined as two or more autosomal monosomies or a single monosomy associated with a structural abnormality, correlates with worse prognosis, including in azacitidine-treated patients [87].

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. For this reason, FISH for 5q31 should be done when cytogenetic analysis yields insufficient adequate metaphases for analysis or when there is an abnormality of chromosome 5. A response to azacitidine correlates with the presence of monosomy 7 [88] but is not confined to such cases. In two series of patients, del(13q) was associated with hypocellular MDS and responsiveness to immunosuppressive therapy [89].

Molecular genetic features

The genetic changes that underlie MDS are multiple and many interact in a given patient. The molecular basis has now been defined to some extent. Characteristically there are multiple genetic lesions, particularly in higher grade MDS. Detailed evaluation is now possible by next generation sequencing (NGS) with a disease-related panel or by SNP (single nucleotide polymorphism) array analysis. Translocations and fusion gene formation are uncommon whereas gene mutations and either amplification or loss of DNA are common. The genetic lesions can be classified as: (i) mutations in genes encoding splicing factors or components (e.g. *SF3B1*, *U2AF1* (*U2AF35*), *SRSF2*, *ZRSR2*, *U2AF2* (*U2AF65*), *SF3A1*, *SF1* and *PRPF40B*); (ii) mutations in genes affecting epigenetic regulation, for example by altering DNA methylation or modifying histones (e.g. *TET2*, *DNMT3A*, *IDH1*, *IDH2*, *ATRX*, *ASXL1*, *EZH2*); (iii) mutations in genes affecting regulation of transcription (e.g. *RUNX1*, *ETV6*, *NPM1*, *GATA2*, *BCOR*); (iv) mutations in genes involved in kinase signalling and downstream pathways (e.g. *JAK2*, *NRAS*, *KRAS*, *MPL*, *CBL*, *KIT*, *FLT3* internal tandem duplication); (v) mutation in genes encoding cohesion complex proteins (*STAG2*, *RAD21*, *SMC3*, *SMC1A*); (vi) loss or mutation of tumour suppressor genes; (vii) loss or mutation of genes required for DNA repair (e.g. *TP53*, *WT1*); and (viii) haploinsufficiency as a result of deletion (e.g. *RPS14*) [90,91]. Additional mutations associated with disease progression may involve growth-controlling genes as well as acquisition of further epigenetic defects.

In a study of 527 patients in whom 111 genes were sequenced, Della Porta *et al.* found oncogenic mutations in 45%. The genes most often mutated were *SF3B1* (26.5%), *TET2* (19.3%), *SRSF2* (13.9%), *ASXL1* (13.9%), *DNMT3A* (9.7%), *EZH2* (7.7%) and *RUNX1* (6.3%) [92]. In two further very large series of patients the genes most often mutated were *SF3B1*, *TET2*, *ASXL1*, *SRSF2*, *DNMT3A*, *RUNX1*, *U2AF1*, *ZRSR2*, *STAG2*, *TP53*, *EZH2*, *CBL*, *JAK2*, *BCOR*, *IDH2* and *NRAS* [93,94].

Mutations that affect splicing are also found in AML with a low blast cell count, suggesting a relationship of these cases to MDS [95].

There is a correlation between genetic abnormalities and cytological features. In a detailed investigation of 245 patients with MDS or MDS/MPN, Malcovati and colleagues [96] found that mutation of *SRSF2*, as well as *SF3B1*, was associated with ring sideroblasts, and coexisting mutations of *SRSF2* and *TET2* correlated with monocytosis. Thrombocytosis was most often associated with mutation in *SF3B1*, *JAK2* or *CALR* while a smaller number of other patients had del(5q), t(3;3) or mutation in *U2AF1*, *MPL* or *SH2B3* [96]. There were also genetic profiles that associated with multi-lineage dysplasia, an

increase of blast cells or both [96]. It has been suggested on the basis of this analysis that a combination of morphology and genetic features could improve the 2008 WHO classification of MDS and MDS/MPN.

Use of SNP analysis or NGS is rapidly expanding from research applications to clinical practice. NGS permits the detection of single base substitutions, the genes most often involved being *TET2*, *DNMT3A*, *RUNX1*, *ASXL1*, *SF3B1* and *SRSF2*. SNP analysis permits the detection of copy number alterations; abnormalities that may be detected include loss of 4q, 5q, 7q, 17p, 20q and 21q and gain of chromosome 8, 4q, 7q, 11q and 17p. Copy-neutral loss of heterozygosity can also be detected, such as loss of both copies of tumour suppressor genes such as *EZH2*, *CBL* and *TP53*. This technique has revealed mutations also in low-risk MDS and these have been found to be of prognostic significance [91].

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;q34.1), abnormalities of 3q21.3 and 3q26.2, and translocations with 11q23 and 21q22.1 breakpoints. In patients whose myeloid cells show loss of all or part of a chromosome, for example −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 [97]. 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* family genes (mainly *NRAS*) and, to a lesser extent in *FMS*, are common in MDS (20–40% and 12–20%, respectively) [98]. *RAS* gene mutations may be induced by irradiation. Mutation or deletion of the tumour suppressor gene *TET2* is found in a fifth to a quarter of patients with MDS and may be homozygous [99,100]; this mutation does not correlate with other disease features and may be an independent good prognostic feature [99,100]. *RUNX1* mutations are common, being found in 10% of patients in one study [101]. *RUNX1* mutations can be induced by both irradiation and anti-cancer chemotherapy but they also occur in *de novo* MDS [101,102]; in *de novo* disease they are associated with higher grade MDS and worse prognosis [101]. *FLT3* mutations (mainly internal tandem duplications) occur in a minority of patients (2% in one series [103]), correlate with trilineage dysplasia and are predictive of transformation to AML [104]. Homozygous mutations in *JAK2*, *TET2*, *CBL*, *CEBPA*, *FLT3* and *EZH2* have been described [91]. *NPM1* mutation was found in 2.8% of 139 patients with MDS, mainly associated with a diploid karyotype, and was not prognostically significant [103]. *DNMT3A* is mutated in 8–15% of patients [105,106]. *IDH1* mutation was found in 3.6% of 193 patients with MDS [107]. 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.

SF3B1 is commonly mutated in patients with ring sideroblasts and is prognostically favourable [108] (see below). Other mutations may be prognostically neutral but in the case of *ASXL1*, *CBL*, *DNMT3A*, *ETV6*, *EZH2*, *IDH1*, *IDH2*, *NRAS*, *PTPN11*, *RUNX1*, *STAG2* and *U2AF1* they are adverse, while *TP53* and *PRPF8* mutations are very adverse [98,105,107,109].

Myelodysplastic syndromes may be associated with mutations of mitochondrial as well as nuclear deoxyribonucleic acid (DNA) [110,111]. 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) [111,112]. However, mitochondrial mutations do not appear to be common [113].

Mitochondrial mutations are likely to precede clonal expansion rather than be the event leading to it [111]. 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 [78].

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 [114] (Fig. 5.17). Peroxidase deficiency of neutrophils can be detected by an abnormal position of the neutrophil cluster; less often peroxidase-deficient neutrophils are present as a distinct population. In other cases the peroxidase activity of dysplastic neutrophils is increased. The presence of blasts cells 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 immature reticulocyte count may be increased even though the absolute reticulocyte count is normal or decreased [115]. 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 [116]. It should, however, be noted that reduced conductivity and scatter have also been reported in transient dysplasia following chemotherapy [116].

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) [117]. An independent study confirmed the adverse

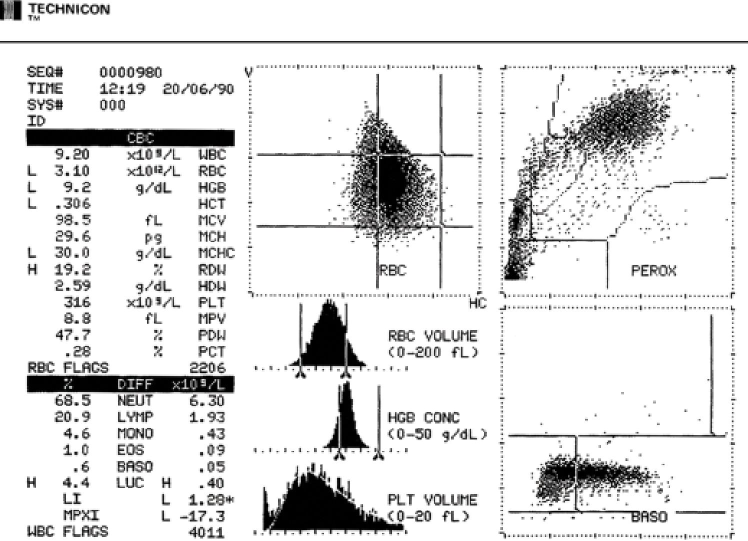


Fig. 5.17 Histograms and scatterplots produced by a Bayer H.1 automated counter in a case of RARS/MDS-RS-SLD showing both macrocytes and hypochromic cells.

prognostic significance of a low platelet mass but not that of a low mean platelet volume [118].

The lymphocyte count may be reduced, with a low count being of adverse prognostic significance [119].

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 and plasma thrombopoietin

Platelet function is often abnormal, particularly in higher grade MDS [120]; defective platelet function can lead to a bleeding tendency, even in patients with a normal platelet count. An increased plasma thrombopoietin correlates with the presence of a PNH clone and a better prognosis [57].

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 [121], and in one retrospective study iron chelation therapy correlated with longer survival [122]. 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 whereas an abnormal pattern is usual in high grade MDS. In one study a marked abnormality of colony growth was observed in refractory cytopenia with multilineage dysplasia with ring sideroblasts but not in RARS without other dysplasia [123]. 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 categories of disease. 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. When acute leukaemia supervenes it may develop within a brief period or there may be a stepwise evolution over many weeks or months. The risk of acute transformation remains constant over time, within risk groups, suggesting that a single critical mutation rather than multiple independent mutations may be causative [124]. 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. Rarely evolution to AML is associated with the acquisition of a t(9;22)/*BCR-ABL1* by the MDS clone [125]. 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 better prognostic categories. Occasional patients with good prognosis subtypes of MDS have died of iron overload.

A number of factors can be correlated with prognosis of MDS (Table 5.5) [62,67,89,119,126–140]. 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 [71,67,79,136,141,142]. 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

Table 5.5 Factors that have been reported to have prognostic significance in the myelodysplastic syndromes.

	Better prognosis	Intermediate prognosis	Worse prognosis
Clinical features	Younger		Older (e.g. >60 years)
	Female		Male
	De novo MDS		Secondary or t-MDS
	No transfusion requirement		Requirement for blood transfusion
		Lower number of transfusions per month	Higher number of transfusions per month
WHO category	MDS with single lineage dysplasia or 5q− syndrome	MDS with multilineage dysplasia or RAEB-1/MDS-EB-1	RAEB-2/MDS-EB-2 or t-MDS
Peripheral blood features			Anaemia (Hb ≤80, ≤90 or ≤100 g/l)
			Neutropenia (neutrophil count <0.5, 0.5–1.0 cf. 1–3 or ≤2.5 cf. >2.5 × 10^9/l)
			Thrombocytopenia (platelet count <20, 20–50, 50–100, 100–150 cf. >150 × 10^9/l)
			Presence of blast cells
			Dyserythropoiesis
			Dysgranulopoiesis
			Dysthrombopoiesis
			Lymphocyte count less than 1.2 × 10^9/l [119]
			Presence of CD34-positive cells
Biochemistry	Normal LDH		Elevated LDH
	Serum ferritin ≤1000 ng/ml		Serum ferritin >1000 ng/ml
Bone marrow aspirate			Increased blast cells (5–10%, 10–20% cf. <5%)
			Increased percentage of CD34-positive cells
			Dyserythropoiesis
			Dysgranulopoiesis
			Dysthrombopoiesis
			Reduced megakaryocytes

(*Continued*)

Table 5.5 (Continued)

	Better prognosis	Intermediate prognosis	Worse prognosis
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	+8, i(17q), –X, trisomy 14, +14q, +19, +21, –21, 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 3q21q26, +11*, +13

CFU-GM, colony-forming units – granulocyte, macrophage; Hb, haemoglobin concentration; LDH, lactate dehydrogenase; MDS, myelodysplastic syndrome; t-MDS, therapy-related MDS; RAEB/MDS-EB, refractory anaemia with excess of blasts/myelodysplastic syndrome with excess blasts.

* Probably poor prognosis rather than intermediate [89].

Derived from references 62, 67, 79, 89, 119, 126–140 and other sources.

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;q21.3), t(6;9)(p23;q34.1), rearrangements with an 11q23.3 breakpoint, 3q21.3q26.2 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 11q23.3 (*KMT2A* rearranged) are specifically associated with evolution to AML with monocytic or granulocytic/monocytic differentiation [77].

Karyotypic abnormalities have been incorporated, together with other variables shown to indicate prognosis, into a number of scoring systems. These include the International Prognostic Scoring System (IPSS) (Table 5.6) [67] and the World Health Organization (WHO) classification-based Prognostic Scoring System (WPSS) (Table 5.7) [143]. Of these, the IPSS has been widely used and validated. The WPSS has likewise been validated (Table 5.8) [143] and has also been found to retain prognostic significance in patients treated by bone marrow transplantation [144]. Modifications of the IPSS cytogenetic

Table 5.6 The International Prognostic Scoring System for myelodysplastic syndrome (MDS).

Score	0	0.5	1.0	1.5	2.0
Prognostic variables					
% 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.

Derived from reference 67.

Table 5.7 The World Health Organization (WHO) classification-based Prognostic Scoring System (WPSS); individual scores are added together to give four prognostic groups with the clinical relevance as shown in Table 5.8.

Score	0	1	2	3
WHO category	RA/MDS-SLD, RARS/MDS-SLD-RS, 5q– syndrome	RCMD/MDS-MLD, RCMD-RS/MDS-MLD-RS	RAEB-1/ MDS-EB-1	RAEB-2/ MDS-EB-2
Karyotype*	Good	Intermediate	Poor	–
Transfusion†	No	Regular	–	–

RA/MDS-SLD, refractory anaemia/myelodysplastic syndrome with single lineage dysplasia; RARS/MDS-SLD-RS, refractory anaemia with ring sideroblasts/myelodysplastic syndrome with single lineage dysplasia and ring sideroblasts; RAEB, refractory anaemia with excess of blasts; RCMD/MDS-MLD, refractory cytopenia with multilineage dysplasia/ myelodysplastic syndrome with multilineage dysplasia; RCMD-RS/MDS-MLD-RS, RCMD and ring sideroblasts, MDS-MLD with ring sideroblasts.

* Defined as in Table 5.6.

† Defined as having at least one red cell transfusion every 8 weeks over a period of 4 months.

Adapted from reference 143.

Table 5.8 Outcome in two cohorts of patients according to the WHO classification-based Prognostic Scoring System (WPPS); 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	$n = 426$	$n = 529$	$n = 426$	$n = 528$
0	103	141	6	3
1	72	66	24	14
2	40	48	48	33
3	21	26	63	54
4	12	9	100	84

n = number of patients.

Adapted from reference 143.

Table 5.9 The International Prognostic Scoring System for myelodysplastic syndrome (MDS), revised (IPSS-R).

Prognostic variable	0	0.5	1	1.5	2	3	4
Cytogenetics*	Very good	–	Good	–	Intermediate	Poor	Very poor
Percentage of BM blasts	≤2	–	>2 but <5	–	5–10	>10	–
Haemoglobin concentration (g/l)	≥100	–	≥80 but <100	<80	–	–	–
Platelet count ($\times 10^9$/l)	≥	≥50 but <100	<50	–	–	–	–
Neutrophil count ($\times 10^9$/l)	≥0.8	<0.8	–	–	–	–	–

* Very good: –Y, del(11q); good: normal, del(5q), del(12p), del(20q), double including del(5q); intermediate: del(7q), +8, +19, i(17q), any other single or double clonal abnormality; poor: –7, inv(3)/t(3;3)/del(3q), double including –7, del(7q), complex with 3 abnormalities; very poor: complex with >3 abnormalities.

Derived from reference 145.

Table 5.10 The International Prognostic Scoring System for myelodysplastic syndrome (MDS), revised (IPSS-R); scores shown in Table 5.8 are summed to give a final score.

Score	Risk
≤1.5	Very low
>1.5 to 3	Low
>3 to 4.5	Intermediate
>4.5 to 6	High
	Very high

Derived from reference 145.

classification have also been proposed with del(11)(q14q23) and del(12p) as single defects being classified as good prognosis, del(7)(q31q35) as intermediate and 3q abnormalities as adverse [79]. This led to development of a revised IPSS scoring system, IPSS-R (Tables 5.9 and 5.10) [145], which has also been validated and shown to be superior to IPSS [140]. The IPSS-R stratification has been reported to be further improved by incorporating information on a monosomal karyotype [87].

Differential diagnosis

The differential diagnosis of MDS includes MDS/MPN and, for high grade MDS, AML. In addition MDS must be distinguished from a heterogeneous groups of conditions that have been designated idiopathic cytopenia of undetermined significance (ICUS) [146,147], idiopathic dysplasia of uncertain significance (IDUS) [147] and clonal haematopoiesis of indeterminate potential (CHIP) [148]. There is some overlap between these three groups of disorders.

CHIP describes a group of individuals who are found to have clonal haemopoiesis without the criteria for MDS or another haematological neoplasm being met. Many appear haematologically normal. Myeloid neoplasm-associated clonal cytogenetic abnormalities such as del(5q), del (11q), del(17p) and del(20q) may be found. Similarly, mutations may be found in genes that are mutated in MDS, such as *DNMT3A*, *ASXL1*, *TET2* and *SF3B1*. CHIP is an age-related phenomenon, found in 5–10% of individuals over the age of 65–70 years [148]. The risk of progression of CHIP to MDS or another specific haematological neoplasm is of the order of 0.5–1% per year [148].

ICUS describes a group of patients with unexplained cytopenia, without an increase of blast cells or sufficient dysplasia to meet the diagnostic criteria for MDS; some subsequently develop MDS. The presence of certain cytogenetic abnormalities that are not considered sufficient for a diagnosis of MDS, specifically trisomy 8, del(20q) and loss of the Y chromosome,

is compatible with this diagnosis. Thirty-five percent of patients with ICUS have a somatic mutation or a cytogenetic abnormality in haemopoietic cells indicative of clonal haemopoiesis [149]; this is higher than the 5–10% frequency of somatic mutation in haematologically normal elderly individuals [148]. Patients with clonal ICUS with a high burden of mutated allele or with two or more mutations have a high risk of progression to MDS and could be regarded as preclinical MDS [150].

IDUS describes a group of patients with unexplained dysplasia but without sufficient cytopenia to meet the criteria for a diagnosis of MDS. Again, some such patients subsequently develop MDS.

Finally, MDS must be distinguished from cytopenia with associated bone marrow dysplasia with a recognizable cause such as congenital dyserythropoietic anaemia, congenital sideroblastic anaemia, vitamin B_{12}, folic acid or copper deficiency, the effects of drugs such as azathioprine, methotrexate, tacrolimus or mycophenolate mofetil or of toxic substances such as arsenic, HIV infection and leishmaniasis.

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 5.11 summarizes the 2008 and 2016 criteria for the WHO categories of *de novo* MDS [6,7,151–164], and Fig. 5.18 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. If a coincidental non-myeloid neoplasm is also present, those cells should be excluded from the differential count for the purposes of determining percentages. 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. Erythroid dysplasia is usually present but is lacking in specificity; in one study, 69% of cytopenic control patients also had erythroid dysplasia in at least 10% of cells [92]. For this reason the authors devised a scoring system to indicate the probability of diagnostically significant erythroid dysplasia but there was still misclassification of 9% of patients who did not have MDS [92]. Granulocytic dysplasia had better specificity but nevertheless 22% of cytopenic control subjects had at least 10% of dysplastic cells; a scoring system improved the specificity to 95% [92]. Megakaryocytic dysplasia was detected in at least 10% of cells in 25% of cytopenic control subjects with a scoring system increasing specificity to 91% [92]. Interestingly, in this study the morphological score for granulocyte and megakaryocytic dysplasia was prognostically significant whereas the score for erythroid dysplasia was not [92]. Further analysis indicated that the 10% cut-off was valid for granulocytic dysplasia while 30% was more informative for megakaryocytic dysplasia [92]. The WHO classification has not adopted a scoring system for significant dysplasia.

Refractory cytopenia with unilineage dysplasia, including refractory anaemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT) – renamed MDS with single lineage dysplasia (MDS-SLD) in the 2016 revision

The great majority of cases that fall into this group have refractory anaemia while a minority have refractory neutropenia or refractory thrombocytopenia [151,152]. There may be cytopenia affecting two lineages but, by definition, not three. This category comprises 5–10%

Table 5.11 The WHO classification of *de novo* myelodysplastic syndromes (MDS) [6,7,151–164].

Type of myelodysplastic syndrome		2016 diagnostic criteria	
2008 terminology	**2016 terminology**	**Peripheral blood findings**	**Bone marrow findings**
Refractory cytopenia with unilineage dysplasia (RCUD) including refractory anaemia (RA), refractory neutropenia (RN) and refractory thrombocytopenia (RT)	Myelodysplastic syndrome with single lineage dysplasia (MDS-SLD)	Anaemia or bicytopenia (not pancytopenia), blasts rarely seen and always less than 1% (1% on two occasions leads to categorization as MDS-U)	Unilineage dysplasia, <5% blasts, <15% ring sideroblasts or <5% ring sideroblasts if *SF3B1* mutation present; blast cells <5%
Refractory anaemia with ring sideroblasts (RARS)	Myelodysplastic syndrome with single lineage dysplasia and ring sideroblasts (MDS-SLD-RS)	Anaemia (rarely bicytopenia), blasts <1%	Dysplasia confined to erythroid lineage, <5% blasts, ≥15% ring sideroblasts or ≥5% ring sideroblasts if there is an *SF3B1* mutation
Refractory cytopenia with multilineage dysplasia (RCMD)	Myelodysplastic syndrome with multilineage dysplasia and ring sideroblasts (MDS-MLD-RS)	Anaemia or bicytopenia, blasts <1%	Dysplasia in ≥10% of cells of two or more lineages, <5% blasts, ≥15% ring sideroblasts or ≥5% ring sideroblasts if there is an *SF3B1* mutation
	Myelodysplastic syndrome with multilineage dysplasia	Cytopenias (bicytopenia or pancytopenia), no or rare blasts (<1%), no Auer rods, <1 × 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)	Myelodysplastic syndrome with excess blasts-1 (MDS-EB-1)	Cytopenias, <5% blasts, no Auer rods, <1 × 10^9/l monocytes	Unilineage or multilineage dysplasia, 5–9% blasts, no Auer rods
Refractory anaemia with excess blasts, 2 (RAEB-2)*	Myelodysplastic syndrome with excess blasts-2 (MDS-EB-2)	Cytopenias, 5–19%* blasts, Auer rods sometimes present, <1 × 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 5.12.
‡ A single additional chromosomal abnormality, excluding monosomy 7 and del(7q), is accepted.

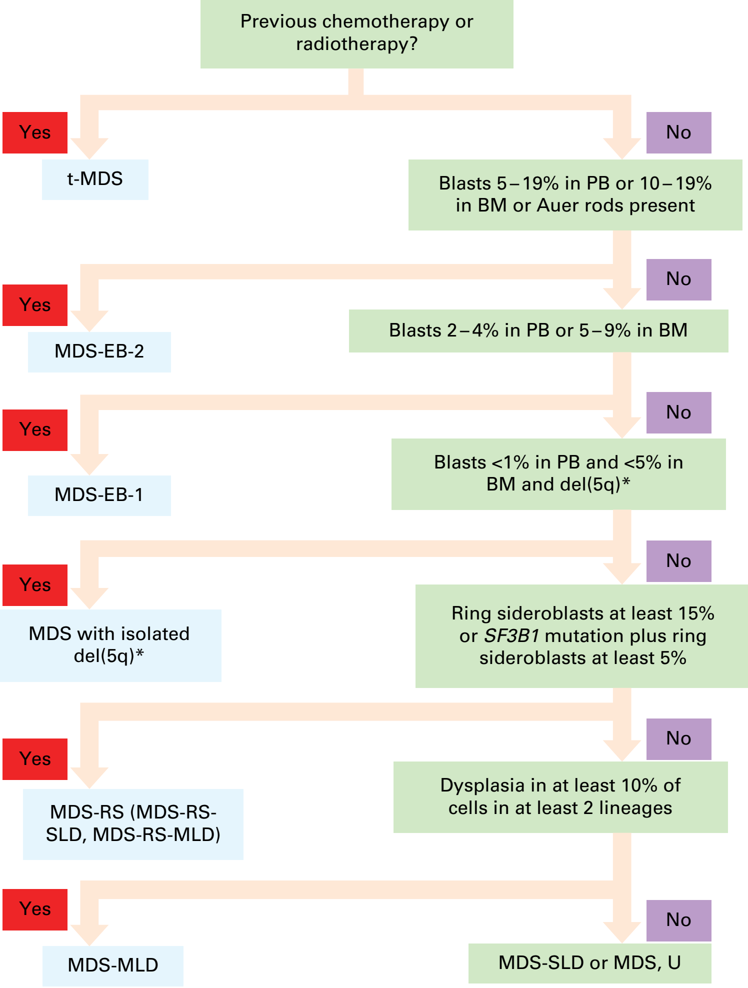


Fig. 5.18 A method of applying the 2016 revision of the WHO classification of MDS in adults. For all categories the monocyte count must be less than 1×10^9/l. t-MDS, therapy-related MDS. *Isolated del(5q) or with a single other abnormality excluding del(7q) and monosomy 7.

of MDS [128]. Significant dysplasia is confined to one lineage, usually but not necessarily the erythroid lineage. Cytopenia and dysplasia usually affect the same lineage but not necessarily. 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. Thrombocytopenia may be cyclical [25]. In some cases of RT, platelet lifespan is somewhat reduced but thrombocytopenia results mainly from 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 seen in megaloblastic anaemia and oval macrocytes are not usual. There may be basophilic stippling. A unique case has been described in which spherocytosis resulted from deletion of

the β spectrin gene [165]. Cases of RN usually have neutrophil dysplasia, and patients with RT may have large or hypogranular platelets. Blast cells are uncommon and, by definition, do not reach 1% on two successive occasions [152].

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 [166] (see Fig. 5.2). 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 or, if an *SF3B1* mutation is present, are less than 5% [152]. 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, for example 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 [79,152]. However, cases that meet the criteria for MDS with isolated del(5q) are excluded from this category. RT is preferentially associated with isolated del(20q), usually del(20)(q11.2) [81]; the dysplasia in these cases may be confined to the erythroid lineage and is not marked [81].

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 [128]. In another series, 8% of patients developed AML and the median survival was 89 months [167]. In this second study the prognosis was significantly worse in refractory thrombocytopenia than in the other two categories, with a median survival of 15.9 months despite a low rate of leukaemic evolution (1/24) [167]. Refractory neutropenia was associated with an intermediate median survival of 35.9 months, with 4 of 23 patients suffering leukaemic transformation [167]. In a third study of 265 patients with refractory anaemia with unilineage dysplasia, median survival was 4.5 years with 8.5% leukaemic transformation at 5 years [168].

Refractory anaemia with ring sideroblasts (RARS) – myelodysplastic syndrome with single lineage dysplasia and ring sideroblasts (MDS-SLD-RS)

In the 2016 WHO revision, RARS of the 2008 classification has been assigned to a category that also incorporates refractory cytopenia with multilineage dysplasia with ring sideroblasts. It has been designated myelodysplastic syndrome with ring sideroblasts and single lineage dysplasia (MDS-RS- SLD) [153,154] (see Table 5.10). Since cases with multilineage dysplasia more often have poor prognosis cytogenetic abnormalities and have a significantly worse survival, it seems more prudent to retain the distinction between these two categories. RARS has ring sideroblasts constituting 15% or more of bone marrow erythroid cells or 5% or more if there is an *SF3B1* mutation [154]. 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 [128]. Significant dysplasia is confined to the erythroid lineage, that is 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 5.19 and 5.20) 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. 5.19). A small number of circulating nucleated red blood cells (NRBC) can often be found, and it may be noted

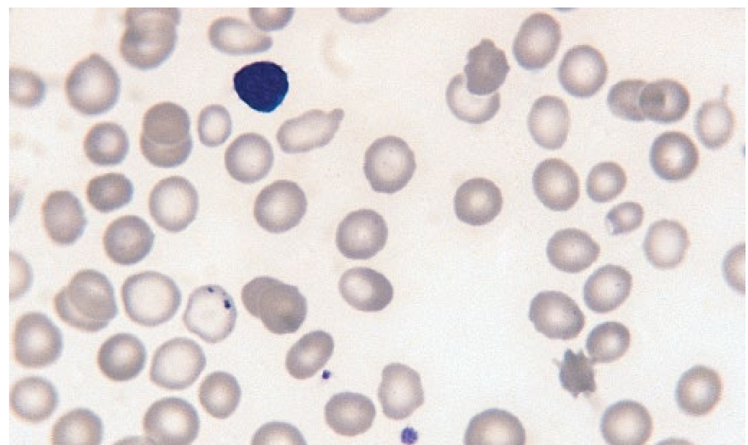
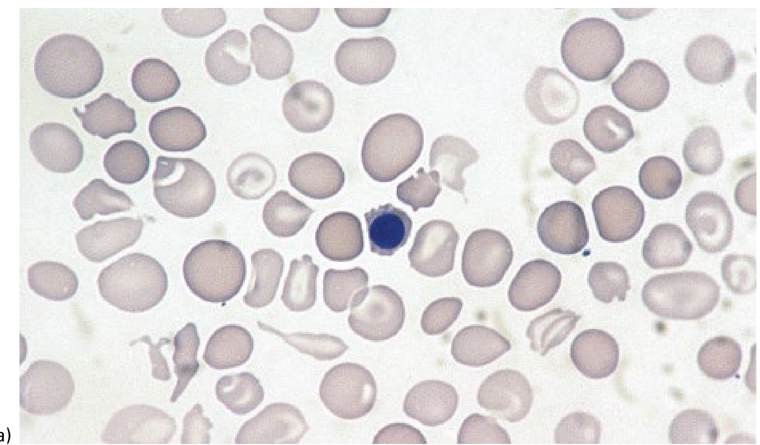
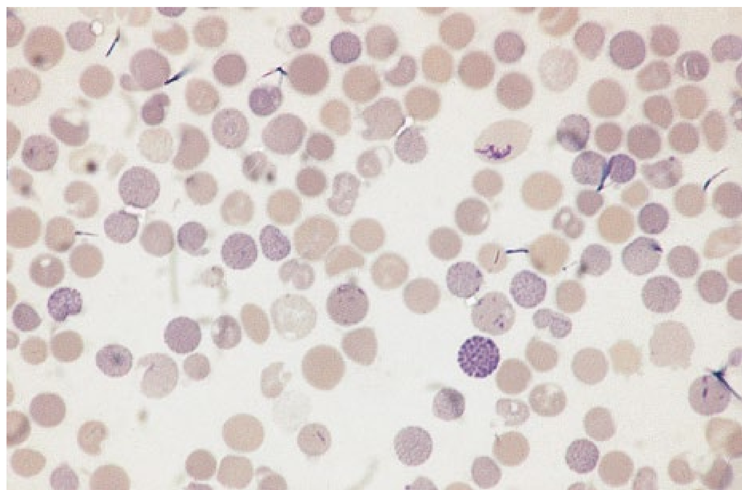


Fig. 5.19 PB film of a patient with RARS/MDS-RS-SLD. The film is dimorphic and one red cell contains Pappenheimer bodies. Poikilocytes including an acanthocyte are present. Pappenheimer bodies are basophilic iron-containing granules, which can be distinguished from basophilic stippling by being larger, more peripherally situated and less numerous within a cell. MGG ×100.



(a)



(b)

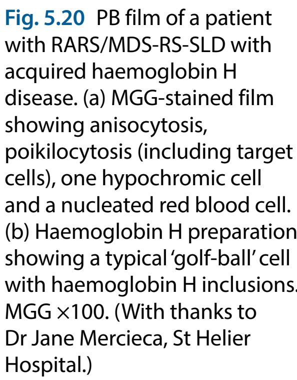
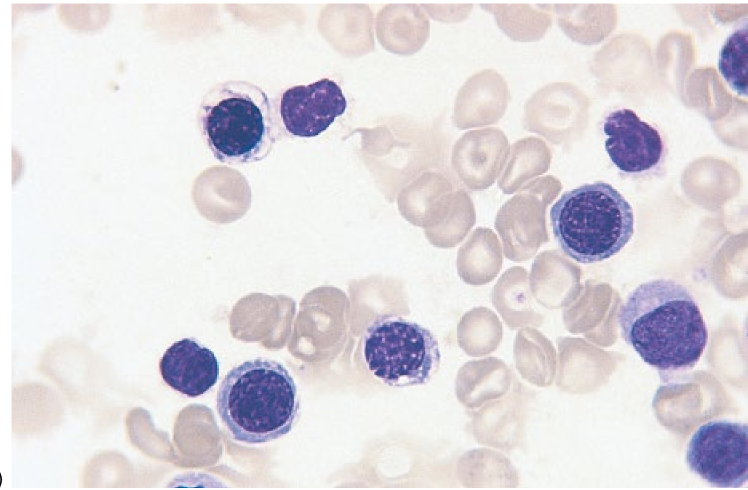


Fig. 5.20 PB film of a patient with RARS/MDS-RS-SLD with acquired haemoglobin H disease. (a) MGG-stained film showing anisocytosis, poikilocytosis (including target cells), one hypochromic cell and a nucleated red blood cell. (b) Haemoglobin H preparation showing a typical 'golf-ball' cell with haemoglobin H inclusions. MGG ×100. (With thanks to Dr Jane Mercieca, St Helier Hospital.)

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). Circulating blast cells are uncommon. By definition, they are less than 1% on two successive examinations [154]. 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. 5.21a). Ring sideroblasts are readily identified on an iron stain (Fig. 5.21b). There is also an increase of other abnormal sideroblasts in which the iron-containing granules are increased in size and

(a)


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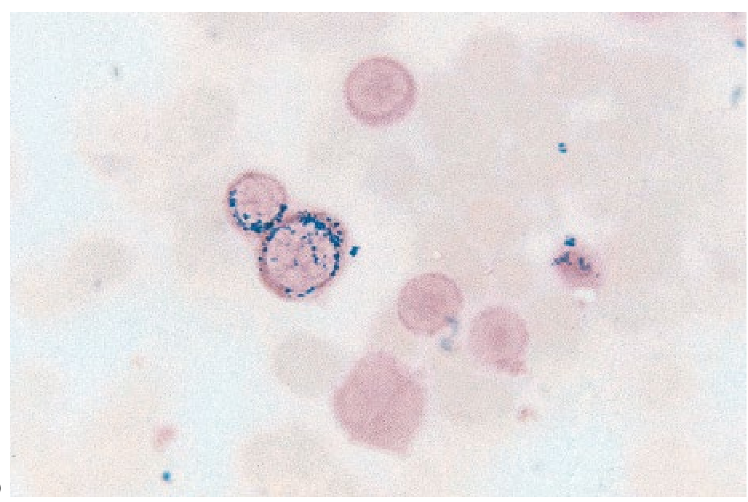


Fig. 5.21 BM aspirate films of a patient with RARS/MDS-RS-SLD. (a) Erythroid hyperplasia with only slight dyserythropoiesis. One erythroblast shows defective haemoglobinization and has basophilic granules within its cytoplasm. MGG ×100. (b) Perls reaction for iron showing two ring sideroblasts. Perls stain ×100.

number but are not disposed in a ring. In RARS, siderotic granules are often present in early 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. The mitochondria also show degenerative changes. 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 [169]. 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 [79,159]. Cases that meet the criteria for MDS with isolated del(5q) are excluded from the RARS category. Mutation in *SF3B1*, a gene encoding an RNA-splicing factor, is a recurrent molecular abnormality found in 70–90% of cases, haploinsufficiency being responsible for the formation of ring sideroblasts [170,171]. Cases with this mutation and with more than 5% but fewer than 15% ring sideroblasts have now been added to this category. There is upregulation of *ALAS2*, encoding δ-amino laevulinate synthase 2 [172], and downregulation of *ABCB7*, a gene encoding a protein involved in transport of iron from mitochondria to the cytoplasm [173]. Cases lacking *SF3B1* mutation may have mutation of other genes involved in RNA splicing including *SRSF2*, *U2AF1* and *ZRSR2* [96,171].

One patient has been described with acquired erythropoietic porphyria associated with (probably radiation-induced) RARS [174]. 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 [175]; 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 [128]. In a study of 206 patients, median survival was 5.2 years with 3.6% leukaemic transformation at 5 years [168]. Although biological characteristics differ, the prognosis of patients with at least 15% ring sideroblasts and no excess of blast cells has been found not to differ from that of similar patients with fewer or no ring sideroblasts [176].

Refractory cytopenia with multilineage dysplasia and ring sideroblasts (RCMD-RS) – myelodysplastic syndrome with ring sideroblasts and multilineage dysplasia (MDS-RS-MLD)

In the 2008 WHO classification, cases that met the criteria for refractory cytopenia with multilineage dysplasia (RCMD) were assigned to this category, whether or not they had a minimum of 15% ring sideroblasts [155]. In contrast, in the 2016 revision, cases with multilineage dysplasia and ring sideroblasts are assigned to the category of MDS-RS, together with cases with single lineage dysplasia; as discussed above, it seems preferable not to merge these groups of cases since they differ in haematological features, cytogenetic abnormalities and prognosis. RCMD-RS is considerably more common that RARS [177].

There may be anaemia, bicytopenia or pancytopenia, bicytopenia being more common than in RARS and pancytopenia excluding categorization as RARS. By definition, there is both dyserythropoiesis with ring sideroblasts and dysplasia in another non-erythroid lineage [154]. Otherwise haematological features at presentation are similar to those of RARS. Evolution to

AML is more common, in one series occurring in 10% of patients in comparison with no leukaemic evolution in RARS [177]. In another series the rate of transformation to AML was 13% [128]

Clonal cytogenetic abnormalities are more common than in RARS, in one series being found in 56% of patients in comparison with 25% in RARS [177]. Poor prognosis cytogenetic abnormalities such as monosomy 7 are also more common than in RARS [178]. Among cases of RCMD-RS, 30–70% have an *SF3B1* mutation in comparison with the 70–90% reported prevalence in MDS-RS-SLD [154,171]. *TP53* mutation may be present in cases that lack an *SF3B1* mutation [171].

Prognosis is worse than that of RARS, with a median survival in one series of 28 months in comparison with more than 72 months in RARS [177]. In another series the median survival was 32 months [128]. When analysis of cases of MDS with 1% or more ring sideroblasts is confined to cases with an *SF3B1* mutation, no survival difference is seen in those with and without multilineage dysplasia [171], indicating the heterogeneity of the RCMD-RS category. Patients with a *TP53* mutation have a worse prognosis [171].

Refractory cytopenia with multilineage dysplasia (RCMD) (without ring sideroblasts) – myelodysplastic syndrome with multilineage dysplasia (MDS-MLD)

In the 2016 revised WHO classification the refractory cytopenia with multilineage dysplasia (RCMD) category no longer encompasses cases with a minimum of 15% ring sideroblasts. 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. 5.22), neutrophils and platelets may show dysplastic features such as the pseudo-Pelger–Huët anomaly (see Fig. 5.1) or hypogranular neutrophils or large or agranular platelets. A unique patient has been described with giant platelets and an acquired Bernard–Soulier type of platelet dysfunction [179]. Acquired elliptocytosis has been described [180]. 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

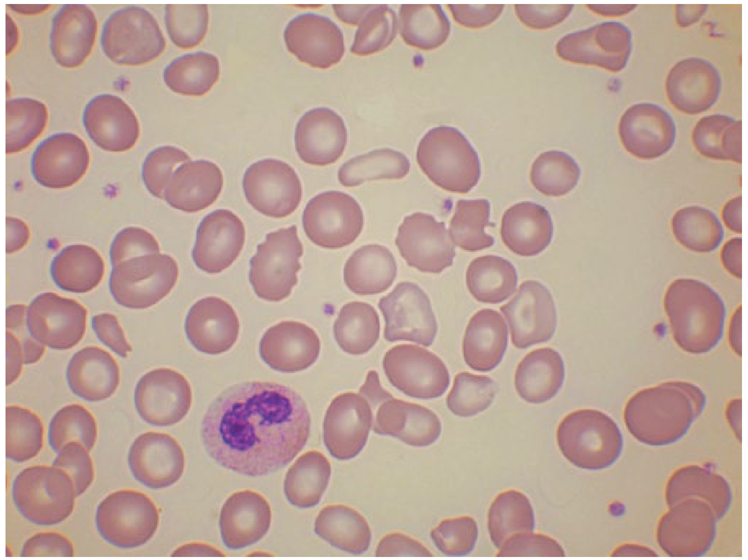


Fig. 5.22 PB film from a patient with refractory cytopenia with multilineage dysplasia (and ring sideroblasts) (RCMD-RS)/MDS-RS-MLD, showing a dysplastic neutrophil, macrocytosis and several hypochromic cells. MGG ×100.

(Figs 5.23–5.25; see also Fig. 5.2). Blasts are less than 5% and some ring sideroblasts may be present. Clonal cytogenetic abnormalities are present in half to three-quarters of patients and may be complex [79,155].

In one series of patients, median survival in those without 15% ring sideroblasts was 33 months, with 10% transformation to AML [28]. In another study of 1912 MDS patients, those with RCMD with or without ring sideroblasts had a median survival of 3 years, with 11.7% leukaemic transformation at 5 years [168].

RCMD in the absence of ring sideroblasts is associated with mutation of genes affecting DNA methylation, RNA splicing (such as *SRSF2*, *U2AF1*, *ZRSR2*), the RAS pathway, and the cohesion complex [96,171]. Mutation of *SF3B1* is less common than in RCMD-RS [171].

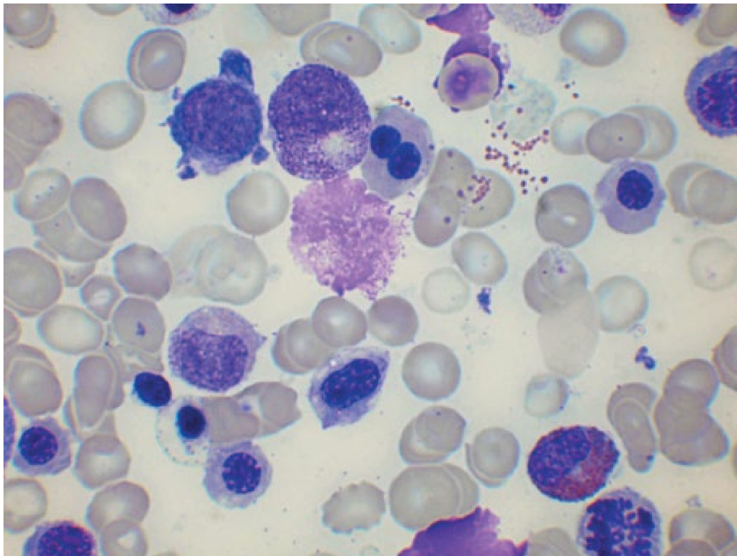


Fig. 5.23 BM aspirate film of a patient with RCMD-RS/MDS-RS-MLD, showing erythroid dysplasia; there is one erythroblast with very scanty cytoplasm (bottom left) and immediately adjacent to it another with almost empty cytoplasm and basophilic granules, some in juxtaposition to the nuclear membrane (likely to be a ring sideroblast). MGG ×100.

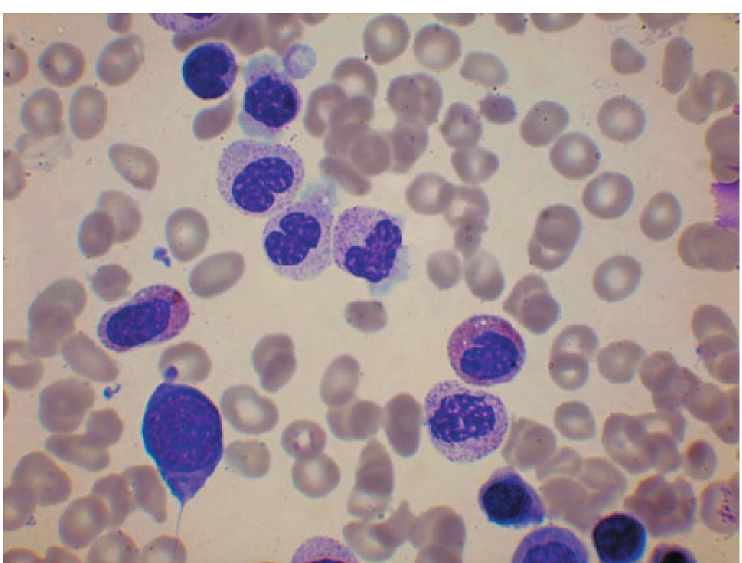


Fig. 5.24 BM aspirate film of a patient with RCMD/MDS-MLD showing one blast cell and a striking pseudo-Pelger–Huët anomaly affecting both neutrophil and eosinophil lineages. MGG ×100.

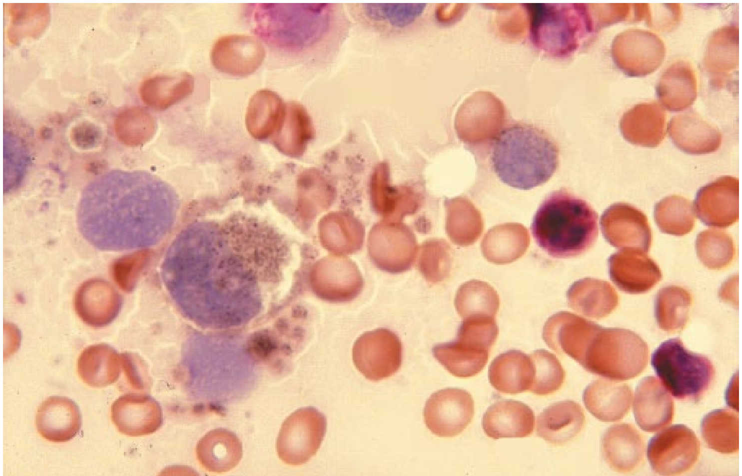


Fig. 5.25 BM aspirate film of a patient with RCMD/MDS-MLD showing platelets, a micromegakaryocyte (left) and a megakaryoblast (right) that are expressing non-specific esterase (brown reaction product); cells of granulocyte lineage are expressing chloroacetate esterase (red reaction product). Double esterase reaction, MGG ×100.

Refractory anaemia with excess of blasts (RAEB) – myelodysplastic syndrome with excess blasts (MDS-EB)

The 2008 WHO classification divides refractory anaemia with excess of blasts (RAEB) into two categories, RAEB-1 and RAEB-2, on the basis of blast numbers [157]; in the 2016 revision this category is redesignated myelodysplastic syndrome with excess blasts (MDS-EB), divided into the same two categories, now designated MDS-EB-1 and MDS-EB-2 [158]. 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 [128]. In the 2016 WHO revision, cases of MDS/AML with greater than 50% bone marrow erythroid cells are categorized according to the blast numbers expressed as a percentage of all bone marrow nucleated cells, and those classified as MDS are designated myelodysplastic syndrome with excess blasts and erythroid predominance (MDS-EB-EP) [158]; this means that some cases that would previously have been categorized as AML now fall into the category of RAEB. However, it should be noted that counting the blast percentage among non-erythroid cells has been shown to give more prognostic information [181]. Other cases have fibrosis and can be designated myelodysplastic syndrome with excess blasts and fibrosis (MDS-EB-F) [158]; bone marrow fibrosis is an adverse prognostic feature [158].

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 [128] (Figs 5.26 and 5.27). There may be ring sideroblasts or Auer rods (RAEB-2). In patients with iron overload, haemosiderin granules are sometimes observed in plasma cells (Fig. 5.28). Significant reticulin deposition occurs in about 15% of cases.

Clonal cytogenetic abnormalities, including trisomy 8, del(20q), 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 [79]. An excess of blast cells in the absence of ring sideroblasts is associated with certain genetic abnormalities, specifically in *RUNX1*, in genes other than *SF3B1* affecting RNA splicing, in RAS pathway genes and in genes encoding the cohesion complex [96]. Mutation of *TP53*, *IDH1*, *IDH2*, *ASXL1* and *CBL* may be present [158].

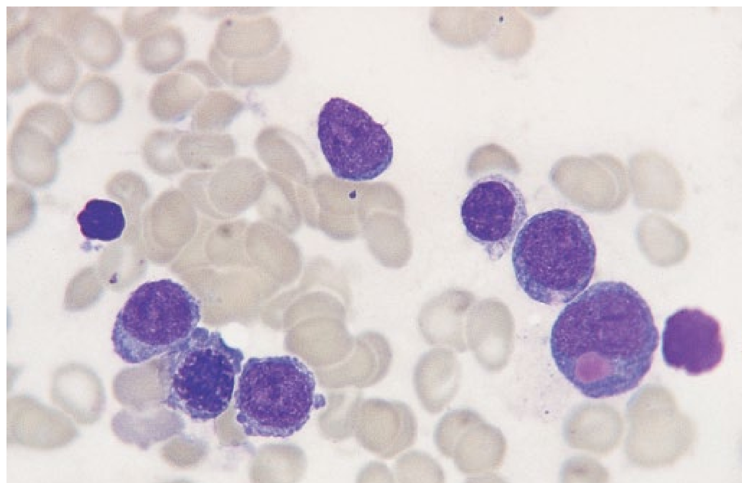


Fig. 5.26 BM aspirate film of a patient with RAEB/MDS-EB showing increased, relatively small blasts and a myeloid precursor with a pseudo-Chédiak–Higashi granule. MGG ×100.

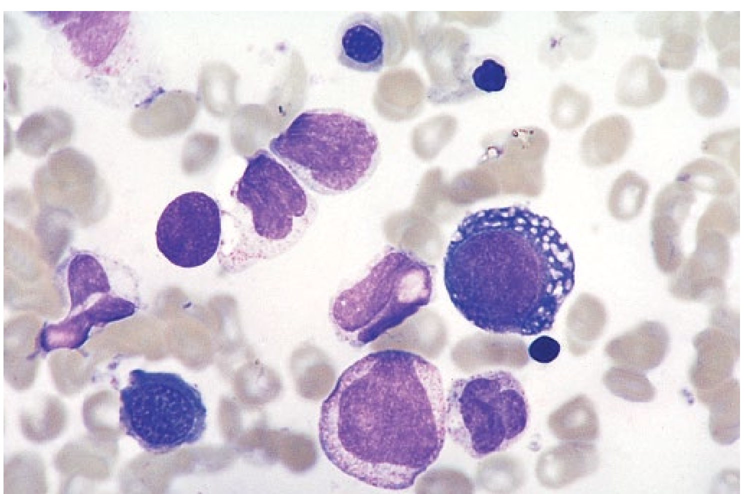
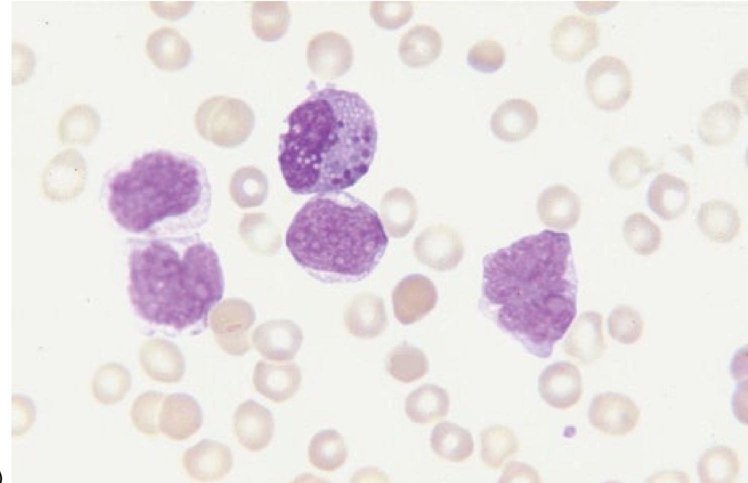
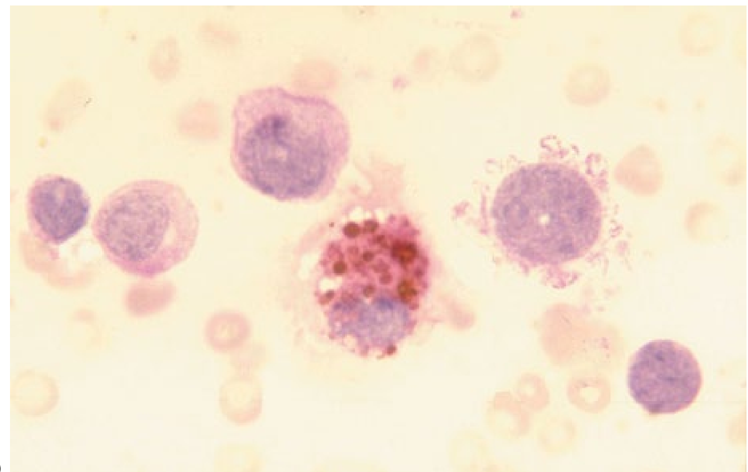


Fig. 5.27 BM aspirate film from a patient with RAEB/MDS-EB showing a blast and a heavily vacuolated red cell precursor. MGG ×100.

Transformation to AML occurs in about a fifth of patients, the others dying of the effects of bone marrow failure. A worse prognosis than in non-RAEB categories and a better prognosis of RAEB-1 than RAEB-2 has been confirmed [39,143]. In one large study the median survival was 16 months in RAEB-1 in comparison with 9 months for RAEB-2 (P = 0.0031) [182]. Similarly, 22% of patients with RAEB-1 suffered transformation to AML in comparison with 40% of patients with RAEB-2 [182]. 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 [182]. 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 [182].

(a)


(b)


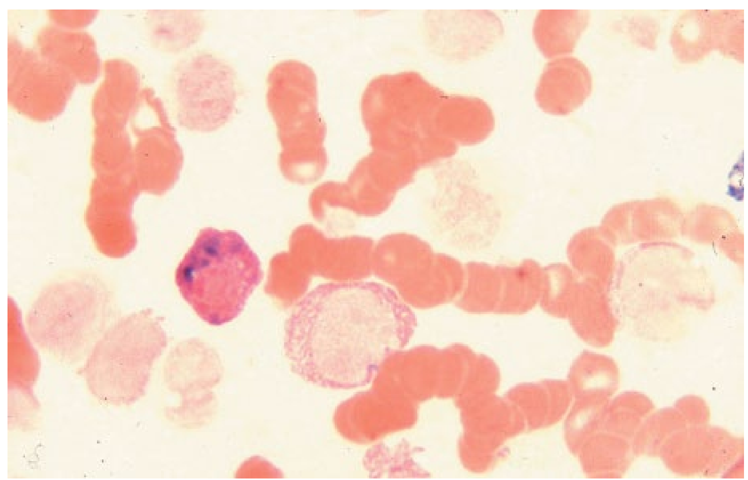
(c)


Fig. 5.28 BM aspirate film from a patient with RAEB-2/MDS-EB-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.

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 [182–186]. The clinical and haematological features are sufficiently consistent that the WHO classification recognizes this as an entity [159]. As defined in the 2008 WHO classification [159], 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%. In the 2016 revision of the WHO classification, cases with a single additional cytogenetic abnormality other than monosomy 7 or del(7q) are also encompassed in this category [160].

Patients are mainly women (75% in one large series of patients) [187], usually middle aged or elderly (median age *c.* 74 years) [187]. The prognosis is relatively good: in one study median survival was more than 5 years and with a low rate of leukaemic transformation (*c.* 6%) [187]; and in a second median survival was 6.2 years with 8.8% leukaemic transformation at 5 years [168]. The rate of leukaemic transformation is not lower than in RA, RARS and RCMD [168]. Age above 70 years and transfusion dependency at diagnosis are associated with a worse survival [187]. Male gender is also associated with a worse prognosis [168]. 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 [10].

The peripheral blood usually shows a macrocytic anaemia (Fig. 5.29), 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 [188]. Erythropoiesis is dysplastic and may be sideroblastic. Megakaryocytes are either 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 [185] (Fig. 5.30); they thus differ from the mononuclear and binuclear micromegakaryocytes (see Fig. 5.2) associated with other forms of MDS, which are no larger than other haemopoietic cells.

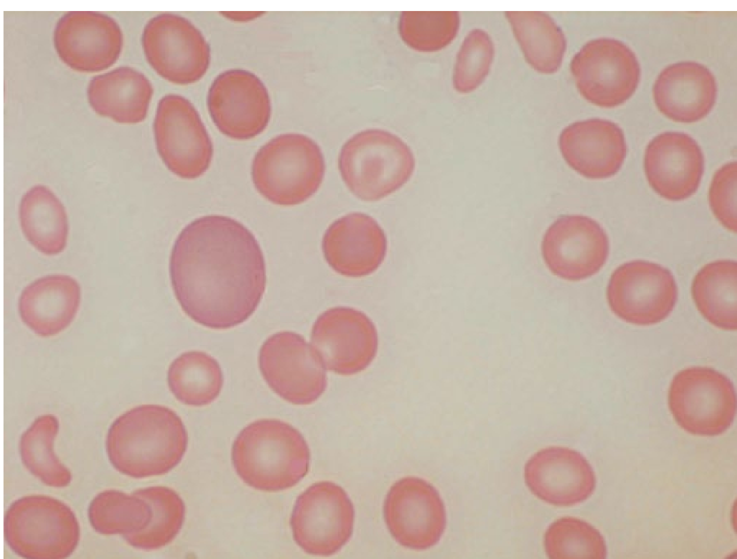


Fig. 5.29 PB film of a patient with the 5q– syndrome showing macrocytes and one target cell. MGG ×100.

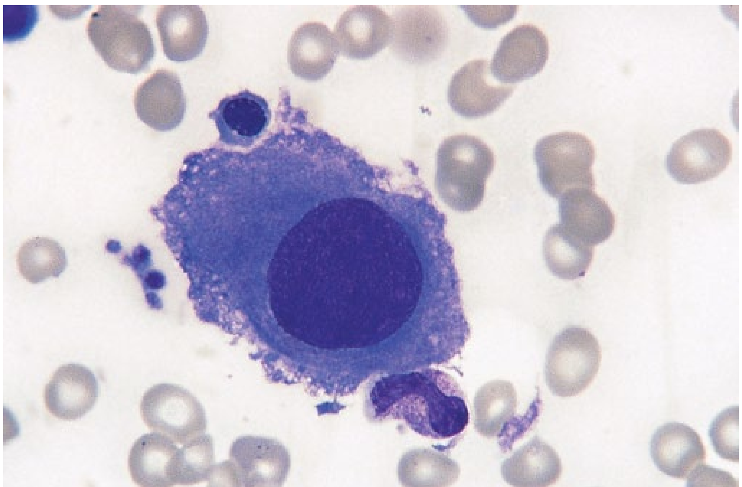


Fig. 5.30 BM aspirate film from a patient with the 5q– syndrome showing a megakaryocyte of normal size but with a hypolobated nucleus. This type of dysplastic megakaryocyte rather than a micromegakaryocyte (see Fig. 5.2) is characteristic of this syndrome. MGG ×100.

Granulocytic dysplasia is present in 10–20% of patients [187,189] and correlates with a worse prognosis [187].

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 genes are *RPS14* at 5q32 [190], the microRNA genes, *MIR145* and *MIR146A*, the tumour suppressor gene, *APC* [191], and *EGR1*. Partial loss of function of *RPS14* can lead to refractory anaemia, downregulation of these two miRNAs can reproduce the phenotype of megakaryocyte dysplasia with increased platelet numbers and subsequent bone marrow failure or AML, and haploinsufficiency of *APC* can lead to ineffective erythropoiesis [191,192]. It may be that haploinsufficiency of these three or four genes, or haploinsufficiency of *RPS14* and *MIR145* [193], is required to produce the phenotype of the 5q– syndrome, and that the 5q– syndrome can be viewed as an acquired contiguous deletion disease. Other genes in the common deleted region include *SPARC*, *RBM22* and *CSNK1A1* [194–196]. The other allele of *CSNK1A1* is mutated in about 5% of patients [197]. 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 [198]. Uniparental disomy involving various chromosomes is common in both the 5q– syndrome and in other MDS with 5q– [199]. A minority of patients with the 5q– syndrome have a *JAK2* V617F, an *MPL* mutation or both [187,200]. *TP53* mutation is present in about 18% of patients and correlates with progression to AML [201]. Patients with ring sideroblasts may have *SF3B1* mutation [171].

In one series of patients classified largely according to the 2008 WHO criteria, the 5q– syndrome comprised around 2% of patients [128]. 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 [128]; 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 [186]. In addition, B lymphocytes are involved in some patients [202]. The detection rate of 5q– is

significantly increased by the use of FISH with a 5q31 probe, with detection of a deletion being seen particularly but not only in patients with failed cytogenetics or abnormalities involving chromosome 5 [203].

This form of MDS is particularly susceptible to treatment with the thalidomide analogue, lenalidomide [204]. Many patients become transfusion independent and some actually become polycythaemic and need venesection [196]. A partial or complete cytogenetic response is common and elevated blast cell counts often fall [196]. The response rate is lower in patients with a *TP53* mutation [201,205]. Strong expression of TP53 in at least 1% of bone marrow cells is indicative of *TP53* mutation and correlates with a higher rate of transformation to AML and a shorter survival [205]. Responses to lenalidomide 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 [196]. The appearance of new, cytogenetically unrelated abnormal clones has been observed during lenalidomide therapy and some patients have suffered transformation to AML. The frequency of AML appears to be higher in patients treated with lenalidomide, particularly but not only in those who do not have a good haematological and cytogenetic response [206]. Bortezomib was reported of benefit in a single patient [207].

Myelodysplastic syndrome, unclassifiable

The WHO classification recognizes some cases of MDS that do not fit the categories described above but nevertheless do have MDS. This category of MDS, unclassifiable (MDS-U), is heterogeneous [161,162]. 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 RCUD/MDS-SLD, RCMD/MDS-MLD, RARS/MDS-RS or MDS with isolated del(5q), but with 1% blast cells in the blood on two consecutive examinations; (ii) resembling RCUD/MDS-SLD or RARS/MDS-RS-SLD or MDS with isolated del(5q) but with pancytopenia rather than unilineage or bilineage cytopenia; and (iii) in the 2016 revision of the WHO classification, also patients having persistent cytopenia with less than 2% blasts in the blood (cf. 1% or fewer in the 2008 classification) 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 (Table 5.12) [32]. Clonal cytogenetic abnormalities were detected in 57% of patients with MDS-U, as defined in the 2001 WHO classification [79].

Table 5.12 Cytogenetic abnormalities providing presumptive evidence of MDS in patients with persistent cytopenia but with insufficient dysplasia to make a morphological diagnosis (2016 revision of WHO classification); abnormalities are shown in the order of their frequency in *de novo* MDS; balanced abnormalities are less common than unbalanced.

Unbalanced	Balanced
−7 or del(7q)	t(11;16)(q23.3;p13.3)
del(5q) or other 5q loss	t(3;21)(q26.2;q22.1)
i(17q) or t(17p)	t(1;3)(p36.3;q21.2)
−13 or del(13q)	t(2;11)(p21;q23)
del(11q)	inv(3)(q21.3q26.2)/(3;3)(q21.3;q26.2)
del(12p) or t(12p)	t(6;9)(p23;q34)
del(9q)	
idic(X)(q13)	

Derived from reference 32.

In addition, there are small numbers of patients with macrocytosis [208] or sideroblastic erythropoiesis [209] who have clonal haemopoiesis but are not anaemic and do not meet the other criteria for inclusion in the WHO categories of MDS. Such cases of ‘refractory macrocytosis’ and ‘refractory sideroblastic erythropoiesis’ are not assigned to the MDS-U category.

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 [210]. 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 and its 2016 revision of a specific category for childhood MDS [163,164]. 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 [163]. 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. Since both dyskeratosis congenita and Fanconi anaemia can be recognized in children who do not have the expected clinical features, these conditions should be specifically excluded before making a diagnosis of RCC. One study of 120 children with a provisional diagnosis of RCC, a normo- or hypocellular bone marrow and a normal karyotype found 17 cases of Fanconi anaemia (based on chromosome breakage studies) and two cases of dyskeratosis congenita [211].

Monosomy 7 is present in about 10% of patients and is predictive of disease progression [210]. 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. 5.1). 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, i(17q) and t(17p), and complex karyotypic abnormalities. There are also several translocations, for example t(11;16)(q23.3;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 under the designation therapy-related myeloid neoplasms [212]. 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 [213].

Other categories of myelodysplastic syndrome

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 [214] 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 [215].

Myelodysplastic syndrome with fibrosis

Some patients with MDS have considerable reticulin deposition. It is important to distinguish these cases from acute megakaryoblastic leukaemia with fibrosis 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 [127]. Fibrosis remains an adverse prognostic factor after haemopoietic stem cell transplantation [216]. As long as a bone marrow aspirate adequate for diagnostic purposes is obtained they can be assigned to a WHO category. Often these cases have excess blast cells, and in the 2016 revision of the WHO classification they are then designated myelodysplastic syndrome with excess blasts and fibrosis (MDS-EB-F) (see above). 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.

Myelodysplastic/ Myeloproliferative Neoplasms

Recognition, nature and epidemiology

The myelodysplastic/myeloproliferative neoplasms (MDS/MPN) are a heterogeneous group of disorders in which there are features of both myelodysplasia and myeloproliferation present at presentation. In the 2016 revision of the WHO classification they are designated as shown in Table 5.13. Patients who present with MDS, mainly refractory anaemia, and subsequently develop features resembling atypical chronic myeloid leukaemia (aCML) [217] are regarded as having evolution of MDS rather than MDS/MPN. Similarly, haematological features resembling aCML occurring as the terminal phase of polycythaemia vera, essential thrombocythaemia and primary myelofibrosis (Fig. 5.31) are regarded as evolution of an MPN rather than MDS/MPN.

Haemopoiesis is effective in at least one lineage and dysplasia is present in at least one lineage. It is usual for at least one lineage to show ineffective haemopoiesis.

Cases with isolated 5q– with thrombocytosis are specifically excluded, these being classified as MDS.

Table 5.13 The WHO classification of myelodysplastic/myeloproliferative neoplasms.

Atypical chronic myeloid leukaemia, *BCR-ABL1*-negative
Chronic myelomonocytic leukaemia
Juvenile myelomonocytic leukaemia
Myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis*
Myelodysplastic/myeloproliferative neoplasm, unclassifiable

* Previously refractory anaemia with ring sideroblasts and thrombocytosis.
Derived from reference 32 and other sources.

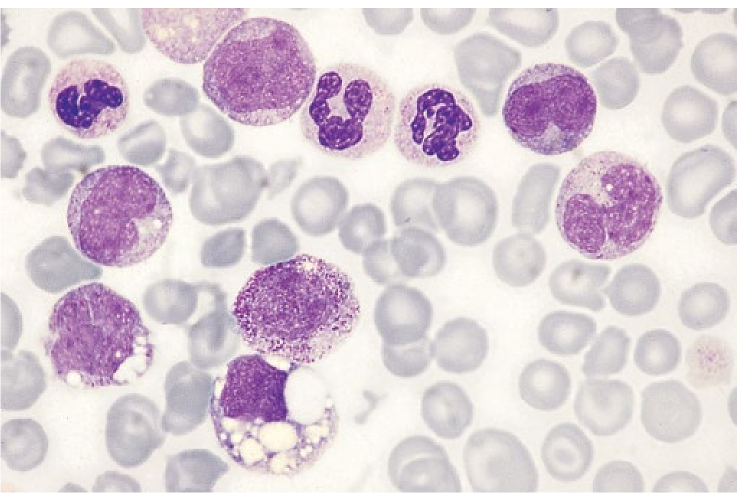


Fig. 5.31 PB film from a patient with chronic myeloid leukaemia evolving from primary myelofibrosis. MGG ×100.

Cytochemistry

A Perls stain is indicated for the identification of ring sideroblasts. Other cytochemical stains may be informative but are not essential for diagnosis.

Immunophenotyping

Immunophenotyping is mainly of use when acute transformation occurs. It has a limited role in chronic phase disease, in helping to distinguish a neoplastic from a reactive condition. Basophils can be specifically identified by flow cytometry or immunohistochemistry using the 2D7 monoclonal antibody, which recognizes a granule protein.

Cytogenetic and molecular genetic analysis

Cytogenetic and molecular analysis may show abnormalities characteristic of MDS (e.g. monosomy 7, del(7q) and mutated *SF3B1*) and others more characteristic of an MPN (e.g. *JAK2* V617F or mutation of *MPL* or *CALR*).

In a detailed genomic investigation, 66/68 patients with MDS/MPN had genomic imbalance, either amplification or deletion of genes, including deletion of *RUNX1* or *CEBPA* [218]. In a more targeted analysis of 187 patients, *RUNX1* mutation was found in 27 patients (14%), *CEBPA* mutation in seven patients (4%), *NPM1* mutation in six patients (3%) and *WT1* mutation in two patients (1%) [218].

The WHO classification of myelodysplastic/ myeloproliferative neoplasms (MDS/MPN)

The 2016 revision of the 2008 WHO classification now includes five MDS/MPN entities, refractory anaemia with ring sideroblasts and thrombocytosis now being separately recognized.

Atypical (Ph-negative) chronic myeloid leukaemia, *BCR-ABL1* negative

Atypical chronic myeloid leukaemia differs from CML clinically, haematologically and genetically. The WHO diagnostic criteria are summarized in Table 5.14 [219,220].

Clinical and haematological features

This condition is much less common than CML. There is a wide age range but patients tend to be elderly, on average 15–20 years older than those with CML. In a series of 65 patients the median age was 72 years (range 42–88) with the condition being twice as common in men as in women [221]. Most patients present with splenomegaly, symptoms of anaemia or, less often, thrombocytopenia and the haematological features of a chronic myeloid leukaemia. Prognosis of aCML is poor, with death resulting from bone marrow failure or transformation to AML. Median survival was 25 months in one large reported series [222] and 12.4 months in another [221].

The peripheral blood shows leucocytosis (Fig. 5.32) with an increase of both neutrophils and their precursors but, in comparison with CML, monocytosis is usually more prominent while eosinophilia and basophilia are less common [223]. Monocytosis is less prominent than in CMML. On average, the WBC is lower than in CML whereas anaemia

Table 5.14 WHO diagnostic criteria for atypical chronic myeloid leukaemia, *BCR-ABL1* negative, and chronic myelomonocytic leukaemia [219,220].

	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 more than 10% of leucocytes
	Less than 20% blasts (plus promonocytes)	Less than 20% blasts (plus promonocytes)
Bone marrow	Hypercellular, 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)(q34.1;q11.2) and *BCR-ABL1* Absence of rearrangement of *PDGFRA*, *PDGFRB*, *FGFR1* Absence of *PCM1-JAK2*, *BCR-JAK2* and *ETV6-JAK2*	

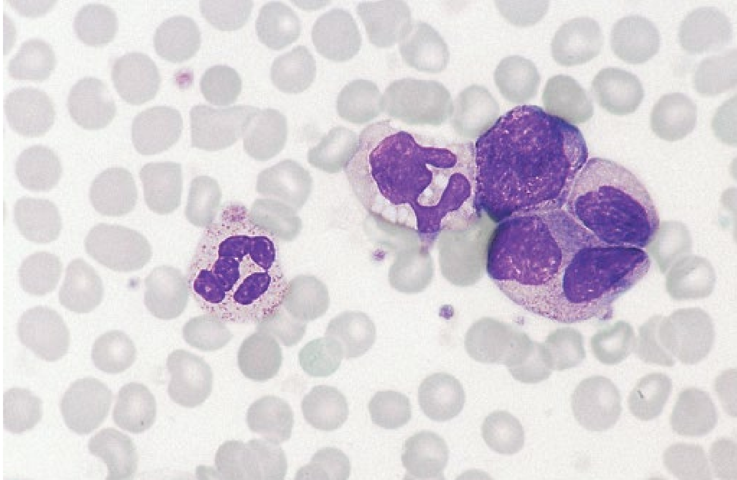


Fig. 5.32 PB film of a patient with atypical (Ph-negative) chronic myeloid leukaemia (aCML) showing a neutrophil, a monocyte, a promyelocyte and two myelocytes, one of which is binucleated. MGG ×100.

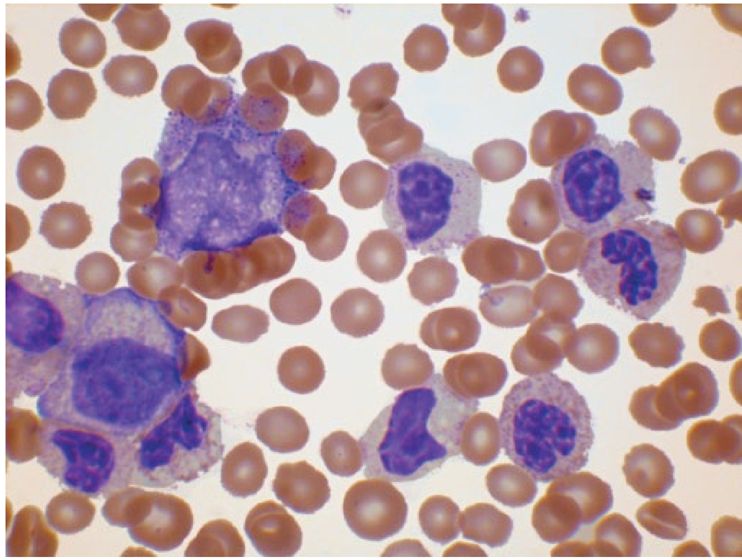


Fig. 5.33 PB film of a patient with aCML showing abnormal chromatin clumping in cells of neutrophil lineage. MGG ×100.

and thrombocytopenia are more common. A minority of patients have thrombocytosis [221]. Dysplastic features are common. Neutrophils may be hypogranular or have nuclei showing an acquired Pelger–Huët anomaly or exaggerated clumping of chromatin (Fig. 5.33) or be hypersegmented [221]. 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, by definition, always less than 20% of circulating leucocytes. The NAP score is commonly decreased but in a minority of patients is increased; it is not of diagnostic value.

The bone marrow findings reflect those in the peripheral blood. There is an increase of granulocytes and precursors but, in contrast to CML, the M : E ratio is usually less than 10 : 1. Basophil and eosinophil precursors are less

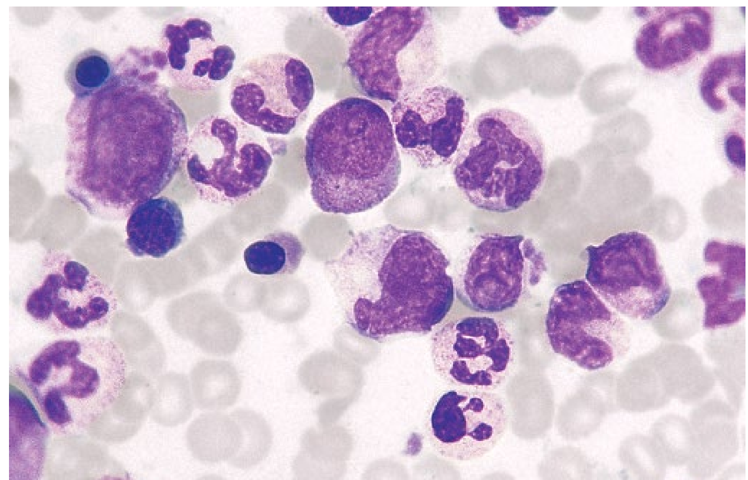


Fig. 5.34 BM film of a patient with aCML (same patient as Fig. 5.32) showing an increase of granulocytes and precursors with dysplastic features; there are also several cells of monocyte lineage. MGG ×100.

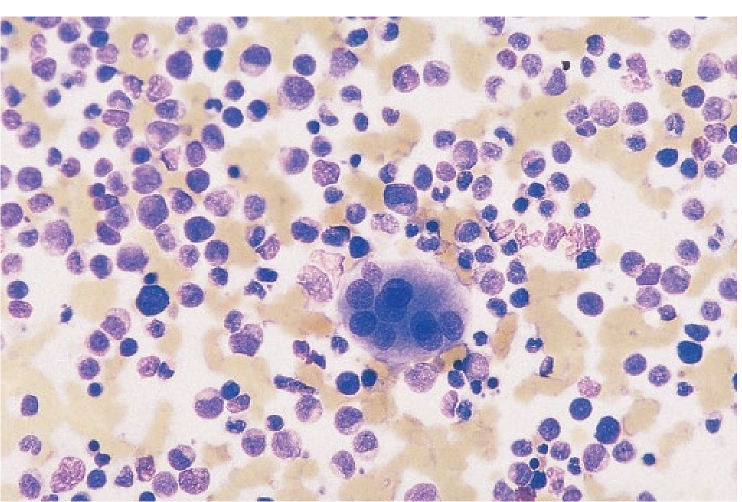


Fig. 5.35 BM film of a patient with aCML showing a multinucleated megakaryocyte. MGG ×40.

often increased whereas monocyte precursors are sometimes more prominent (Fig. 5.34). Megakaryocytes are reduced in about a third of patients and may be dysplastic (Fig. 5.35). Blasts may be somewhat increased but blasts plus promonocytes are less than 20%. Significant fibrosis is present in approaching a third of patients [221].

Atypical CML can terminate in blast crisis. This is usually a myeloid crisis but occasional lymphoid blast crises have been observed [224] suggesting an origin in a pluripotent haemopoietic stem cell. In one large series of patients 22 of 55 (40%) suffered transformation to AML [222], and in another 24 of 65 (37%) [221].

Although aCML is usually readily distinguished from chronic phase CML it can be difficult to make the distinction from CML in early transformation 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, i(17q), monosomy 7, del(7q), del(20q) and complex rearrangements are relatively common [221,225]. There is no t(9;22)(q34.1;q11.2) or *BCR-ABL1* fusion and, by definition, no rearrangement of *PDGFRA*, *PDGFRB* or *FGFR1* and no *PCM1-JAK2*. The somatic mutations most often identified are in *SETBP1*, *ASXL1*, *TET2*, *EZH2*, *NRAS* and *KRAS* [226]. A *SETBP1* mutation was found in about a quarter of patients in one series [227], in a quarter of 74 patients in another [228] and in 15% of 20 patients in a third [225]. In the latter series, the *SETBP1* mutation coexisted with a *CSF3R* mutation in one patient, and in another seven patients (35%) there was a *CSF3R* mutation without *SETBP1* mutation [225]. In one study inactivating mutations of *CBL* were found in 12 of 52 patients (34%) and of *TET2* in 13 of 38 (34%) [229]. In another study, however, *TET2* mutation was uncommon [230]. Inactivating *EZH2* mutations were reported in 9 of 70 patients (13%) [231]. Mutation of *NRAS* or *KRAS* is found in about a third of patients. *IDH2* mutations have been described. A *CSF3R* mutation was found in 8 of 18 patients in one series [232] but in none of 27 in another [221] and is considered not to be characteristic of aCML. *ETNK1* was mutated in 14% of 29 patients in one series of patients [233]. *JAK2* V617F was found in 3 of 42 patients in one series [221]. *CALR* mutation is not generally a feature [221].

Problems and pitfalls

Because of the therapeutic implications, it is important not to misdiagnose CML 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 have rearrangement of *PDGFRB*, who are likely to respond to targeted therapy. 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 at least 1×10^9/l, no Ph chromosome or *BCR-ABL1* fusion gene, and fewer than 20% blasts plus promonocytes in the blood and marrow [220]. Cases with rearrangement of *PDGFRA*, *PDGFRB*, *FGFR1* or with *PCM1-JAK2*, *BCR-JAK2* or *ETV6-JAK2* are also excluded. In the 2016 revision of the WHO classification it is recommended that CMML be further divided into: CMML-0, with blast cells being less than 2% in the peripheral blood and less than 5% in the bone marrow; CMML-1, with blasts being 2–4% in the blood or 5–9% 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 5.14. A prognostic scoring system has been proposed and validated, based on FAB and WHO subtypes, cytogenetic characteristics and transfusion dependency [234]. Some cases of CMML are therapy-related, with a median latency of 6 years [235]. Therapy-related cases tend to have prognostically worse cytogenetic abnormalities and have a worse overall survival [235]. In the 2016 revision of the WHO classification, therapy-related cases are classified with other therapy-related myeloid neoplasms.

Clinical and haematological features

Patients are mainly elderly (median age 65 to 75 years) with a male predominance (1.7–2:1) [236,237]. The incidence in the USA has been estimated at 0.37/100 000/year [238], and in the Netherlands the age-standardized incidence rate is 0.4/100 000/year [237]. The 5-year relative survival rate is 20% [237]. Patients usually present with symptoms of anaemia, bleeding or infection, or with clinical features suggesting myeloproliferation (e.g. splenomegaly with or without

hepatomegaly, weight loss, night sweats). However, in many patients the diagnosis is an incidental one when a blood count is done for another reason. A minority of patients have pleural, pericardial or peritoneal effusions, synovitis, lymphadenopathy or skin infiltration [239–241]. Skin infiltration can represent blastic transformation, blastic plasmacytoid dendritic cell neoplasm or a proliferation of mature plasmacytoid dendritic cells [242]. Rarely gum hypertrophy occurs [239]. Serum lysozyme is usually increased and urinary lysozyme is sometimes increased. Rare cases of renal failure may be related either to elevated urinary lysozyme [241] 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 5.36 and 5.37) with the monocyte count being at least 1×10^9/l.

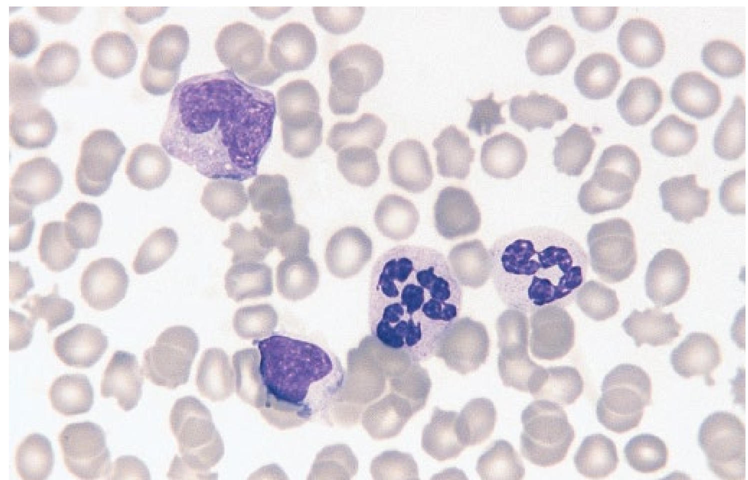


Fig. 5.36 PB film of a patient with CMML showing a monocyte, a lymphocyte and two neutrophils, one of which is a macropolycyte. The red cells are poikilocytic and platelet numbers are reduced. MGG ×100.

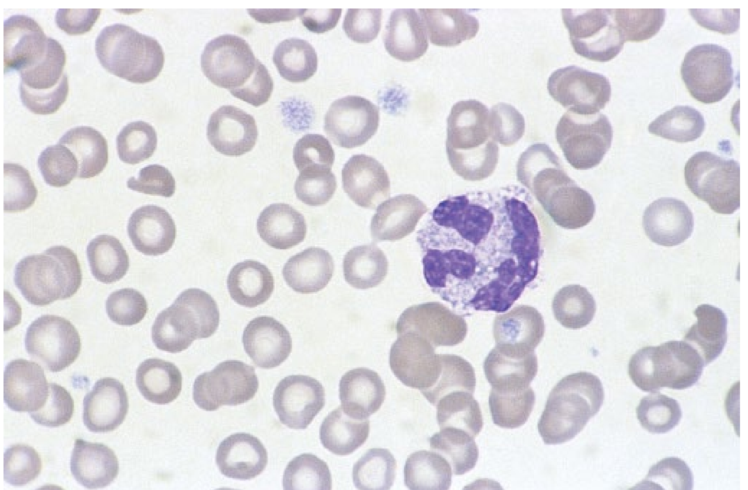


Fig. 5.37 PB film of a patient with CMML showing a binucleated macropolycyte. There is also anisocytosis, poikilocytosis, hypochromia and thrombocytopenia. MGG ×100.

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. Rarely microcytosis is due to acquired haemoglobin H disease resulting from an *ATRX* mutation [29]. 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 5.38 and 5.39) is usually hypercellular. Immature monocytes

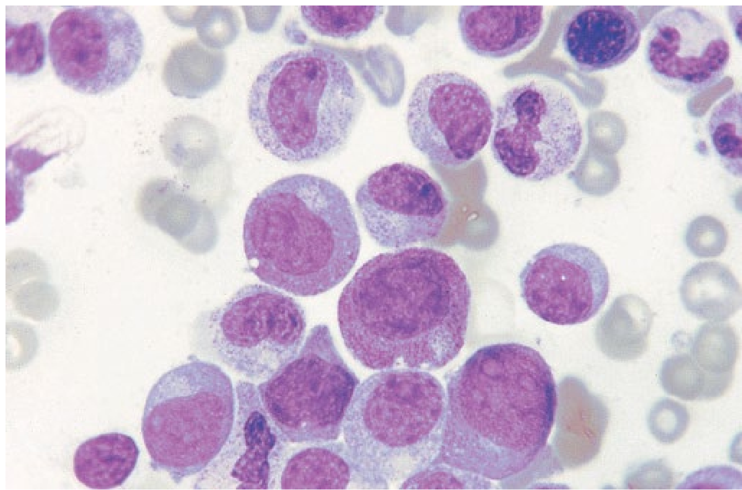


Fig. 5.38 BM film from a patient with CMML showing predominantly an increase in granulocytes and their precursors. MGG ×100.

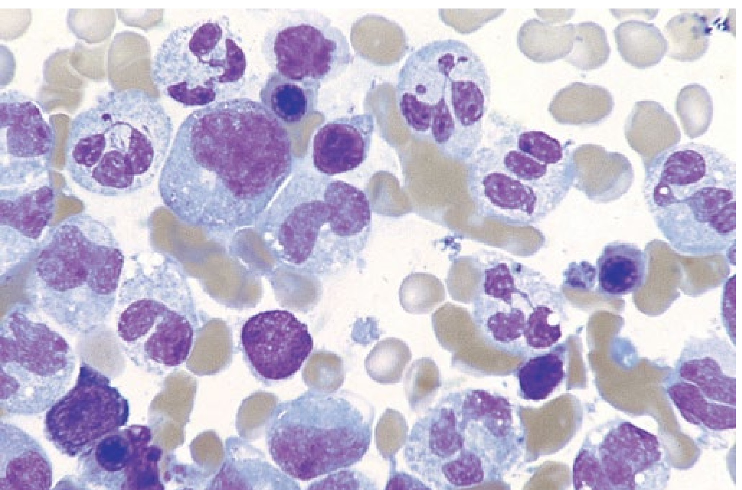


Fig. 5.39 BM film of a patient with CMML showing granulocytic dysplasia. The neutrophils are hypogranular and in addition there is a defect of nuclear lobulation with unusually long filaments separating lobes, an appearance showing some similarities to the inherited condition known as myelokathexis. MGG ×100. (With thanks to Dr A. Copplestone, Plymouth.)

may be prominent with relatively few mature monocytes. In some patients there is marked granulocytic hyperplasia with monocyte precursors being inconspicuous. An unusual reported observation is of rectangular PAS-positive crystals, and oval and irregular inclusions in bone marrow cells [243]. 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 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 (identified by CD123 immunohistochemistry), and sometimes such infiltration is paratrabecular [244]. Reticulin deposition is sometimes increased. Blasts (plus promonocytes) are up to 20%.

The immunophenotype of peripheral blood monocytes often shows aberrant features such as reduced expression of CD13, CD14, CD15, CD36 and HLA-DR, aberrant expression of CD2 and CD56 [245,246] and asynchronous expression of antigens, such as CD34 expression on maturing cells. A population of monocytes with reduced CD11c expression is present in more than half of patients [247]. Immunophenotyping can contribute to distinguishing CMML from reactive monocytosis, for example when increased expression of CD56 is present or when there are more than two aberrancies in antigen expressions [248] or when more than 94% of monocytes are CD14+CD16− [249]. A small population of circulating plasmacytoid dendritic cells can sometimes be identified on immunophenotyping [244].

The prognosis of CMML has been very variable between different series of patients. 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 [248]; in a 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% [250]. 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 [251]. 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 [252]. CMML-2 has a worse prognosis than CMML-1, again reflecting the prognostic significance of increased blast cells. In a fourth series of patients univariate analysis showed an adverse prognosis associated with a WBC above 13×10^9/l, a lymphocyte count above 2.5×10^9/l, a platelet count less than 100×10^9/l and trilineage myelodysplasia, but only a higher lymphocyte count was significant on multivariate analysis [253]. In another study, worse overall survival was independently associated with WBC above 15×10^9/l, platelet count less than 100×10^9/l and anaemia (Hb less than 110 g/l in a man, less than 100 g/l in a woman) [254]. In a series of 274 patients, blast transformation occurred in 13%, with circulating blast cells and female gender being predictive [255]. An increase of CD34-positive cells in trephine biopsy sections is also prognostically adverse (although monoblasts may not express CD34).

Cytogenetic abnormalities and some molecular abnormalities are of prognostic significance (see below). A number of prognostic scoring systems have been devised [254,255].

Cytogenetic and molecular genetic features

An abnormal karyotype is found in 20–40% of patients. Clonal cytogenetic abnormalities most often described are trisomy 8, monosomy 7, del(7q), abnormalities of 12p including del(12p), del(20q), del(5q), trisomy 10, del(11q), add(17p), der(3), trisomy 19 and trisomy 21 [255,256]. In a study of 414 Spanish patients with successful cytogenetic analysis at diagnosis, 27% had a cytogenetic abnormality [257], and in a French–US study of 409 patients the proportion was 30% [256]. The presence of any cytogenetic abnormality other than –Y was associated with a worse prognosis [257]. Three risk groups could be defined: (i) low risk, normal or –Y only; (ii) high risk, trisomy 8, abnormality of chromosome 7 or complex (more than three abnormalities); and (iii) intermediate, other cases [257]. A subsequent US study suggested that trisomy 8 was more appropriately assigned to the intermediate risk group, and found patients with more than three cytogenetic abnormalities to have a worse prognosis than those with three abnormalities [258]. A French-US study found normal, –Y only and der(3q) only to be good risk, and complex and monosomal karyotype to be poor risk, with trisomy 8 alone again falling into the intermediate category [256]. Acquisition of a cytogenetic abnormality during the course of the illness is also prognostically adverse, particularly when the abnormality is complex [259].

A variety of molecular genetic abnormalities can be present and can coexist; such abnormalities can be identified in 90% of patients. The genes most often mutated are *TET2*, *SRSF2*, *ASXL1*, *RUNX1*, *NRAS* and *KRAS* [226]. Abnormalities fall into four broad groups:

1) Epigenetic regulators (*EZH2*, *ASXL1*, *TET2*, *DNMT3A*, *IDH1* and *IDH2*). Uniparental disomy of 7q was found in 26% of patients in one series, and in 41% of these patients there was a homozygous mutation of *EZH2* [260]. An *ASXL1* mutation was found in 43% (17/39) of patients in one study [261], in 40% in another having adverse prognostic significance [254], and in 37% in another, again with adverse prognostic significance [256]. Mutations or deletion of *TET2* were found in two of nine patients in one series [99], 6 of 17 in a second [230], 13 of 26 in a third [229], 29 of 69 in a fourth [262] and 44 of 88 in a fifth [263]. *TET2* mutations are often biallelic, mainly as a result of acquired uniparental disomy [230]. It is possible that *TET2* mutations are prognostically adverse [263].
2) Spliceosome components (*SF3B1*, *SRSF2*, *U2AF1*, *ZRSR2*, *SF3A1*, *PRPF40B*, *U2AF2* and *SF1*). Mutually exclusive mutations in one of the genes controlling the spliceosome (*SRSF2*, *SF3B1* or *U2AF35)* are found in about half of patients and do not appear to be of prognostic significance [264]; *SF3B1* mutation correlates with the presence of ring sideroblasts, with an abnormal karyotype and with der(3q) [256]. *SRSF2* mutation correlates with a normal karyotype [256].
3) DNA damage response genes (*TP53*).
4) Tyrosine kinases and transcription factors (*JAK2*, *KRAS*, *NRAS*, *CBL*, *FLT3* and *RUNX1*). *RAS* gene mutations (*NRAS* or *KRAS*) were found in 4 of 35 patients in one series [265] and in 25 of 65 patients in another [250]. In another series of 70 patients, *KRAS* was mutated in 24% of patients, *NRAS* in 17% and both in one patient [266]. In the same series of patients, *BRAF* was mutated in 7% of patients, somewhat more frequently when *NRAS* and *KRAS* were wild type [266]. *RUNX1* mutations are common, in one series being found in 6 of 27 patients [267] and, in another series, in 30 of 81 patients [268]. *CBL* inactivating mutations were found in 10 of 78 patients (13%) in one study [229]; these mutations may also be homozygous due to uniparental disomy [229]. Reduced expression of *TRIM33* (also known as *TIF1G*) due to hypermethylation, seen in a third of

patients, is another probable pathogenetic mechanism [269]. *JAK2* V617F mutation is found in about 10% of patients and is associated with a more proliferative phenotype [226]. *JAK2* V617F and a *CBL* mutation can coexist [270]. In a study that included patients with coexisting systemic mastocytosis and CMML the *KIT* D816V mutation was found in leukaemic cells of eight of nine patients with these two diagnoses [271].

5) Other. Mutations of genes involved in the Notch pathway (*NCSTN*, *APH1*, *MAML1* or *NOTCH2*) were found in 5 of 42 patients in one study [272]; the contribution to leukaemogenesis may be loss of tumour suppressor function of the Notch pathway. A *SETBP1* mutation was found in 14.5% of 152 patients in one series [227] but in only 3/82 (4%) in a second [228].

Data summarized by Bacher *et al*. [246] from a number of studies, including those already cited, permit a calculation of the prevalence of mutations as follows: *ASXL1* 44%, *TET2* 40%; *RAS* genes (*NRAS* > *KRAS*) 28%, *RUNX1* 24%; *CBL* 14%, *JAK2* V617F 10%, *IDH* 8%, *NPM1* 5% and *PTPN11* 3.5%. Some mutations coexist (e.g. *RUNX1* with *NRAS* or *KRAS*) whereas others appear not to [246]. Malcovati *et al.* found coexisting mutations of *TET2* and *SRSF2* to be particularly characteristic [171].

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. It is essential to recognize the dispersed chromatin pattern that is a feature of promonocytes to make the distinction from immature monocytes. Cytogenetic analysis is essential in any patient with CMML who has eosinophilia, in order not to miss patients who have rearrangement of *PDGFRB*.

Juvenile myelomonocytic leukaemia

Juvenile myelomonocytic leukaemia (JMML) is a Ph-negative MDS/MPN of children typically characterized by mutation of one of a group of genes involved in RAS pathway signalling. Causative mutations may occur in a pluripotent lymphoid–myeloid haemopoietic stem cell [273]. JMML is rare, the incidence being about 1.8 cases/1 000 000/year [274]. The WHO criteria for this diagnosis are summarized in Table 5.15 [275]. Predisposing conditions include neurofibromatosis type 1 (*NF1* mutation), Noonan syndrome (*PTPN11* mutation or less often *SOS1*, *RAF1*, *KRAS* or *NRAS* mutation) and *CBL* mutation-associated syndrome. It should be noted that a significant proportion of children with Noonan syndrome have abnormal haemopoiesis resembling JMML that is transient [276]; 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 [274,277]. As in the case of Down syndrome, the transient nature of the abnormality does not exclude the possibility that it is neoplastic in nature.

Clinical and haematological features

Onset is usually in infancy (median age 2 years, three-quarters of cases under 3 years of age) and there is a male predominance. Common clinical features include fever, anaemia, hepatomegaly, marked splenomegaly, lymphadenopathy, tonsillar enlargement, an eczematous or maculopapular rash, xanthomas and a bleeding tendency. Infections, including tonsillitis and bronchitis, are common. Intestinal infiltration can lead to diarrhoea, and pulmonary infiltration to cough and tachypnoea [278]. 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. Children with *CBL* mutation may have developmental delay, pigmented skin lesions, impaired growth, cardiac abnormalities and cryptorchidism [279].

The peripheral blood (Fig. 5.40) shows leucocytosis, neutrophilia and prominent monocytosis. Granulocyte precursors including

Table 5.15 WHO criteria for a diagnosis of juvenile myelomonocytic leukaemia.

All of the following	Monocyte count at least $1.0 \times 10^9/1$ Blasts plus promonocytes less than 20% in peripheral blood and bone marrow Splenomegaly No Ph chromosome of *BCR-ABL1*
PLUS	
One of the following	Somatic mutation in *PTPN1, KRAS* or *NRAS* Clinical diagnosis of neurofibromatosis type 1 or *NF1* mutation Germline *CBL* mutation and loss of heterozygosity for *CBL*
OR	
In the absence of the above genetic features	Either monosomy 7 or other chromosomal abnormality OR At least two of the following Haemoglobin F increased for age Myeloid or erythroid precursors in peripheral blood GM-CSF hypersensitivity on culture Hyperphosphorylation of STAT5

Ph, Philadelphia chromosome; GM-CSF, granulocyte–monocyte colony-stimulating factor.
Adapted from reference 275.

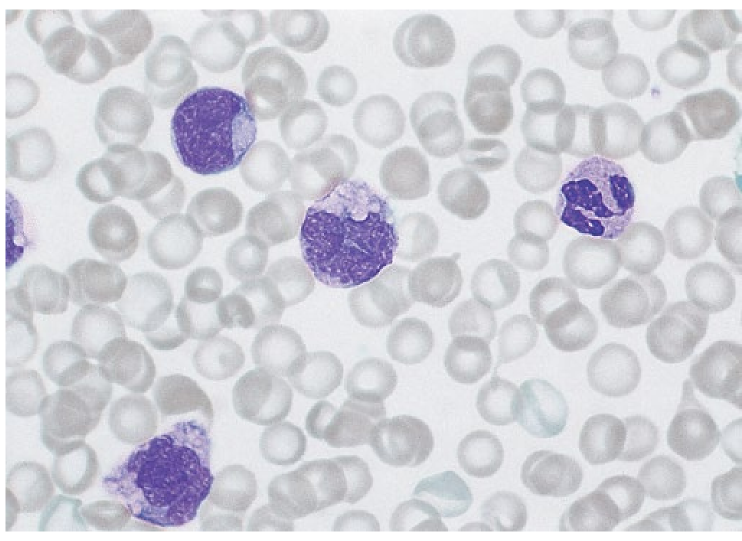


Fig. 5.40 PB film of a child with JMML showing a blast cell and several cytologically abnormal monocytes. MGG ×100.

blasts are often present, with blasts being less than 2% [278]. 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. Microcytosis can be the result of an acquired β thalassaemia phenotype [280].

The bone marrow is hypercellular with increased granulopoiesis and usually an

Peak name	Calibrated area %	Area %	Retention time (min)	Peak area
F	82.0*	----	1.21	950224
P3	----	1.5	1.69	20487
A0	----	28.3	2.56	383310

Total area: 1354022

F concentration = 82.0*%

A2 concentration = %

*Values outside of expected ranges

Analysis comments:

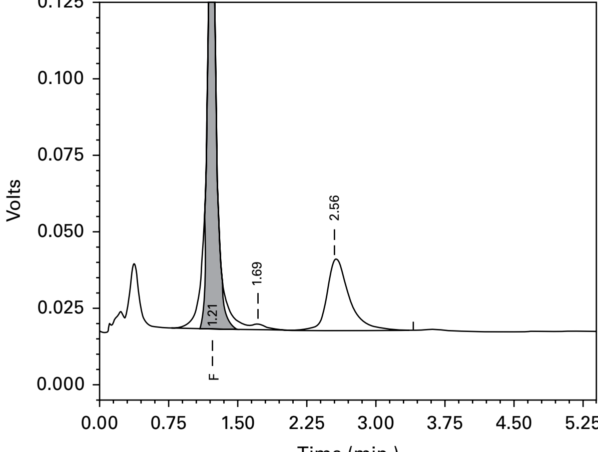


Fig. 5.41 High performance liquid chromatography on a Bio-Rad Variant II instrument in a child with JMML showing an increase of haemoglobin F to 70–80% of total haemoglobin and a total absence of haemoglobin A_2, both these abnormalities being characteristic of fetal erythropoiesis.

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. 5.41). 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 haemoglobin F percentage is significantly increased, the oxygen dissociation curve is left shifted. It should be noted that haemoglobin F is elevated at birth in normal infants and 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. Increased haemoglobin F occurs in patients who lack monosomy 7 and is related to overexpression of *LIN28B* [281]. The concentration of serum immunoglobulin is increased in 50–80% of patients, and some have a positive direct antiglobulin test or antinuclear or other autoantibodies. Serum lysozyme is increased.

Spontaneous colony growth from peripheral blood or bone marrow cells [282], 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, about 10% [283,284], 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 1 year [285]. 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) [275,286]. Prognosis is also dependent on the specific genetic defect, being particularly poor with *PTPN11* and *KRAS* mutations [278].

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. Blast transformation is often associated with monosomy 7 or a complex cytogenetic abnormality [284].

Molecular genetic abnormalities involve the RAS pathway, with mutation in *NRAS*, *KRAS*, *RRAS*, *RRAS*2, *PTPN11*, *NF1* or *CBL* [287]. More than one of these closely related mutations occur together in about 11% of patients [287]. *NRAS* and *HRAS* mutations are present in about a quarter of children [274,286]. Rarely there is a constitutional *NRAS* mutation [288]. In an exceptional case with a somatic *KRAS* mutation, JMML was preceded by RAS-associated lymphoproliferative disorder [289]. *NRAS*-associated cases have sometimes shown spontaneous resolution [278]. Children with neurofibromatosis constitute around 11% of cases of JMML [274]; they often show loss of the normal *NF1* allele, which may be the result of acquired uniparental disomy [290–292] or of somatic mutation of the normal allele [**292**]. JMML may be the first manifestation of neurofibromatosis [287]. 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 [277]. 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 [274]. A *CBL* mutation, most often homozygous due to acquired uniparental disomy [270], has been reported in 7–18% of patients [270,293]. Spontaneous resolution can occur in JMML associated with homozygous *CBL* mutation [278,294].

Mutations in *SETBP1*, *JAK3* and *SH2B3* can occur as second events [287]. *ZRSR2* is sometimes mutated in association with RAS pathway mutations [287]. An *ASXL1* mutation was found in 2 of 49 patients in one series [295], in 3 of 68 patients in a second [293] and in 8 of 100 patients in another [287]; *ASXL1* mutation often coexists with a RAS pathway mutation [287] and has been associated with an older age of onset [293].

In children presenting under the age of 2 years and who have a somatic mutation in *NRAS*, *KRAS* or *PTPN11*, analysis of Guthrie card blood spots shows that the mutation is often already present at birth [296].

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 [273]. Osteopetrosis can also simulate JMML. It should be noted that neonates and infants with Noonan syndrome can have a syndrome resembling JMML that resolves over months or years [278]; this is believed to be polyclonal and if this is so it is correctly regarded as a leukaemoid reaction. A distinction should also be made from the condition designated RAS-associated autoimmune leucoproliferative disorder (RALD), which is a clonal disorder associated with *NRAS* or *KRAS* mutation but which has a more indolent clinical course [297]. Peripheral blood

immunophenotyping, demonstrating an increase of B cells and the presence of CD10-positive B cells, can aid in making the distinction [297].

Refractory anaemia with ring sideroblasts and thrombocytosis – myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis

Cases of refractory anaemia with ring sideroblasts and thrombocytosis (RARS-T)/myelodysplastic/myeloproliferative neoplasm with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T) is recognized as a specific entity in the 2016 revision of the WHO classification [298]. There are at least 15% ring sideroblasts and the platelet count is at least 450×10^9/l. Blast cells are less than 1% in the peripheral blood and less than 5% in the bone marrow. The blood film is dimorphic with a population of hypochromic microcytes; there may be macrocytosis and platelets may be large. Cases do not meet the criteria for any other MDS or MDS/MPN. Patients are on average similar in age to patients with RARS but older than those with essential thrombocythaemia [299]. The prognosis is better than that of RARS but worse than that of essential thrombocythaemia [300]. There is a thrombotic risk similar to that of essential thrombocythaemia [300]. Progression to AML sometimes occurs.

A founding mutation in *SF3B1*, a common mutation in RARS, has been reported in 65–90% of patients with RARS-T in different series, with an associated subclonal mutation, mainly *JAK2* V617F (typical of polycythaemia vera and common in essential thrombocythaemia and primary myelofibrosis) but sometimes *MPL* or *CALR* mutation [300,301]. In three series of patients *JAK2* V617F was reported in 43% [300], 77% of patients [301] and 79% [299] of cases. *TET2* mutations may also be found [299]. Cytogenetic analysis is usually normal [301].

By analogy with other categories of MDS/MPN it seems preferable that this category is restricted to a *de novo* presentation with both myelodysplastic and myeloproliferative features; however, the WHO classification permits a prior diagnosis of RARS [298]. Documentation of evolution of RARS with del(11q) to RARS-T associated with acquisition of del(20q) and *JAK2* V617F [302] and other similar cases [300] indicates how this overlap syndrome may arise. However, it is likely that in some instances a *JAK2* or other MPN-associated mutation occurs first [301].

Myelodysplastic/myeloproliferative neoplasm, unclassified

This category includes all cases of MDS/MPN that do not meet the criteria for the categories already defined above [303]. RARS-T is no longer included in this category. Diagnosis requires that in addition to meeting the criteria for one of the categories of MDS, there is also a platelet count of at least 450×10^9/l or a WBC of at least 13×10^9/l.

Clinical and haematological features

There is usually leucocytosis, often anaemia, and nearly a third have thrombocytosis; dysgranulopoiesis is present in half [221]. Thrombocytopenia can occur and, uncommonly, neutropenia [221]. In one series of patients the median age was 71 years (range 55–88) with a higher incidence in males [221], and hepatomegaly or splenomegaly was found in a fifth of patients [221]. The median survival was 21.8 months with progression to AML occurring in a quarter of patients [221].

Cytogenetic and molecular genetic features

Cytogenetic abnormalities observed include trisomy 8, monosomy 7, del(7q), i(17q) and complex abnormalities [221,303].

JAK2 V617F mutation was found in 10/52 patients (19%) [221]. A *SETBP1* mutation was found in 3/30 patients [228]. *KRAS* and *NRAS* mutations may be found but *CSF3R* or *CALR* mutation is not a recognized feature [221].

Conclusions

The classification of MDS and MDS/MPN is based on morphology but the contribution of molecular genetics is increasing. The recognition of MDS with isolated del(5q) and of the association of an *SF3B1* mutation with RARS and RARS-T are the first steps towards a more molecularly-based classification. Since MDS is highly heterogeneous and molecular changes are very complex it may be that further advances in classification will be slow. However, molecular analysis is likely to increasingly influence not only diagnosis and classification but also patient management.

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6

Chronic Myeloid Leukaemias

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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 2016 revision of the 2008 WHO classification of these various chronic myeloid leukaemias is summarized in Table 6.1 [1–3]. Those that are classified as MDS/MPN are discussed in Chapter 5.

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; it can be used to identify cells of basophilic lineage and during acute transformation has a role in identifying the lineage of blasts.

Chronic myeloid leukaemia, *BCR-ABL1*-positive

Chronic myeloid leukaemia (CML), *BCR-ABL1*-positive, is a disease entity with specific haematological, cytogenetic and molecular genetic features, associated with a Philadelphia (Ph) chromosome and a *BCR-ABL1* fusion gene. Since in the 2016 revision of the WHO classification, chronic myeloid leukaemia is the preferred designation [4], I have adopted this term. However, it is not really very satisfactory, since it can also be used as a generic term to include all chronic myeloid leukaemias, a use analogous to the use of the term 'acute myeloid leukaemia'. Its adoption for this specific entity necessitates the clumsy addition of '*BCR-ABL1*-positive' in order

Table 6.1 Classification of the chronic myeloid leukaemias and related conditions, based on the 2016 revision of the 2008 WHO classification [1–3].

Myeloproliferative neoplasms
Chronic myeloid leukaemia, Philadelphia chromosome positive (t(9;22)(q34.1;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/lymphoid neoplasms with eosinophilia and abnormalities of *PDGFRA*, *PDGFRB* or *FGFR1* or with *PCM1-JAK2*
Myeloid (and lymphoid) neoplasms with *PDGFRA* rearrangement
Myeloid (and lymphoid) neoplasms with *PDGFRB* rearrangement
Myeloid (and lymphoid) neoplasms with *FGFR1* rearrangement
Myeloid (and lymphoid) neoplasms with *PCM1-JAK2*

to make a distinction from atypical chronic myeloid leukaemia (aCML). Alternative designations are 'chronic granulocytic leukaemia', 'chronic myelogenous leukaemia' and 'chronic myelocytic leukaemia'.

The incidence is of the order of 1.6/100 000/year [5], 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 the tyrosine kinase inhibitor (TKI), imatinib, develop Ph-negative high grade myelodysplastic syndrome (MDS) or acute myeloid leukaemia (AML) [6]; the interpretation of this event is more problematical. Chronic myeloid leukaemia may develop more frequently than would be expected by chance in patients with other MPN (see below).

The chronic phase of chronic myeloid leukaemia

Clinical and haematological features

Chronic myeloid leukaemia is predominantly a disease of adults. The usual clinical presentation is with splenomegaly, hepatomegaly, symptoms of anaemia, early satiety and discomfort in the region of the spleen, and systemic symptoms such as sweating and weight loss. Less frequent are priapism and retinal haemorrhages. In a survey of 430 patients, presenting symptoms were fatigue and lethargy (33%), bleeding (21%), weight loss (20%), splenic discomfort (19%) and sweats (15%) [7]. The spleen was palpable in 76% of patients (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 CML has developed in a patient with a pre-existing Ph-negative MPN [8]. Spontaneous regression during pregnancy has been described, the white blood cell count (WBC) falling from 93 to 25×10^9/l and the platelet count from above 600 to 180×10^9/l [9].

The peripheral blood usually shows anaemia and leucocytosis with a very characteristic differential count [10] (Fig. 6.1). The two predominant cell types are the myelocyte and the mature neutrophil (Fig. 6.2). Earlier granulocyte precursors are also 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

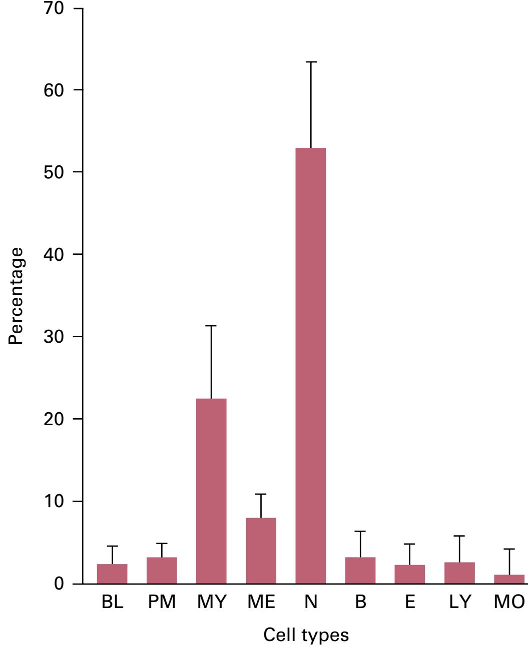


Fig. 6.1 Diagrammatic representation of the typical differential count in 50 untreated cases of chronic myeloid leukaemia (CML) (all demonstrated to be Philadelphia (Ph) positive). Each differential count was of 1500 cells. BL, blasts; PM, promyelocytes; MY, myelocytes; ME, metamyelocytes; N, neutrophils; B, basophils; E, nucleated erythroid cells; LY, lymphocytes; MO, monocytes. The mean and the standard deviation are indicated. (Modified from reference 10.)

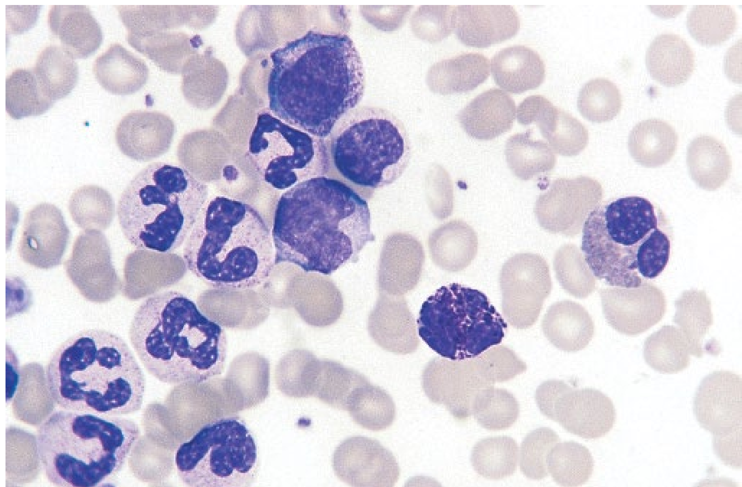


Fig. 6.2 Peripheral blood (PB) film of a patient with CML showing two promyelocytes, a myelocyte, an eosinophil, a basophil and numerous neutrophils and band forms. May–Grünwald–Giemsa (MGG) ×100.

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 WBC correlates with the degree of splenomegaly, and both correlate inversely with the haemoglobin concentration (Hb) [7]. 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 [7]. Very rare patients have eosinophilia without neutrophilia [11]. 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 CML (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 (1–2%) with a p190 BCR-ABL1 protein – more characteristic of Ph-positive acute lymphoblastic leukaemia (ALL) than of CML – have a more prominent relative and absolute monocytosis while those with the very rare p230 BCR-ABL1 protein may have a neutrophilic variant of CML [12]. The p190 protein is associated with a relatively poor response to imatinib and probably also to other TKIs [13].

In untreated CML, there is usually a progressive rise in the WBC. Rare patients show cyclical changes in the count, for example from 30 to 500×10^9/l with a periodicity of about 50 days, suggesting a partially intact negative feedback mechanism [14]; in this reported patient there was a similar cycle in the platelet count but 1 week in advance of the changes in the WBC [14]. In some patients with cyclical changes, haematological remissions are sometimes prolonged although haemopoietic cells remain Ph positive [15]. The NAP score may cycle inversely to the white cell count [15].

Following successful imatinib therapy, the leucocytosis and thrombocytosis resolve and there may be anaemia and transient neutropenia and thrombocytopenia; anaemia may become chronic [16].

The bone marrow is intensely hypercellular with a marked increase in granulopoiesis (Fig. 6.3) 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

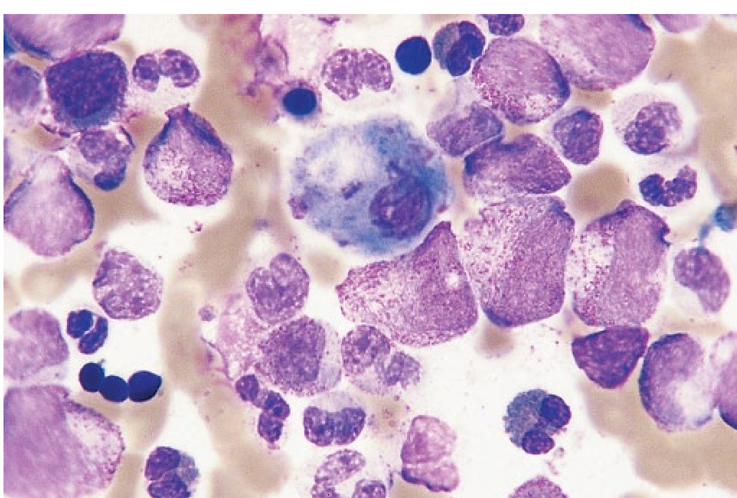


Fig. 6.3 Bone marrow (BM) film of a patient with CML showing increased granulocytes and precursors and a phagocyte containing cellular debris. MGG ×100.

at presentation. An increase of reticulin is much more frequent.

Several staging systems have been proposed for the further categorization of patients with CML, in order to give an indication of prognosis. The most widely used have been the Sokal score [17], developed for busulfan-treated patients, and the Hasford score [18], developed for interferon-treated patients [19]. Both these continue to give useful prognostic information in patients treated with TKIs. The European Treatment and Outcome Study (EUTOS) score, based only on the basophil percentage and spleen size below the costal margin, has been developed specifically for TKI-treated patients and has been validated [20].

Cytogenetic and molecular genetic features

Chronic myeloid 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 [21] 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)(q34.1;q11.2), with the derivative chromosome 22q− being the previously reported Philadelphia chromosome [22]. 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 22q11.2 with some of the sequences of the *ABL1* oncogene, which have been translocated from 9q34.1. A chimaeric 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, which is not always transcribed and, since a protein product has not been identified [23], 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 CML. 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 [24]. There are also patients with otherwise typical CML 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 9q34.1, at 22q11.2 or on a third chromosome [23,25]. Cases without a Ph chromosome but with *BCR-ABL1* rearrangement are classified as CML. 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 [12]. These show different disease associations (Table 6.2). Patients presenting with isolated thrombocythaemia usually have p210.

Fluorescence *in situ* hybridization (FISH) demonstrates that a significant minority of patients with CML have a large deletion of chromosome 9 material adjacent to the breakpoint on the derivative chromosome 9 [26]. 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) [27], resistance to interferon therapy [28] and, in the past, a considerably worse prognosis [26–29]. The adverse effect of deletion of der(9) is confined to those patients in whom the deletion spans the breakpoint [30] and is negated by the use of imatinib therapy [31]. Chromosome 9 deletions appear to occur at the time of the initial translocation and are more common in patients with variant translocations [29]. 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 [32]. 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 [32]. Patients may have additional cytogenetic abnormalities present at diagnosis, this correlating with a slower response and a lower rate of cytogenetic and molecular response to imatinib but without a statistically significant reduction in long-term outcome [33].

There is some evidence that the translocation that leads to *BCR-ABL1* formation may not be the first molecular event in CML. 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 [34].

Table 6.2 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 CML About a third of cases of Ph-positive ALL
$p190^{BCR\text{-}ABL1}$	m-bcr	A minority of cases of CML, 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 CML, or CML with marked thrombocytosis

ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CML, chronic myeloid leukaemia; Ph, Philadelphia chromosome.

The characteristic chromosomal rearrangement of CML can be detected by conventional cytogenetic analysis (Fig. 6.4), 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. 6.5. 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. 6.6). 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 6.7 and 6.8. Patients in whom CML is suspected should have conventional cytogenetic analysis performed, and if a classical t(9;22) is not detected should be further studied by FISH. RT-PCR should be performed to permit future monitoring. 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 CML. 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, CML can be monitored by conventional cytogenetic analysis, FISH and RT-PCR and its modifications [35], particularly by real time quantitative PCR (RQ-PCR). 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

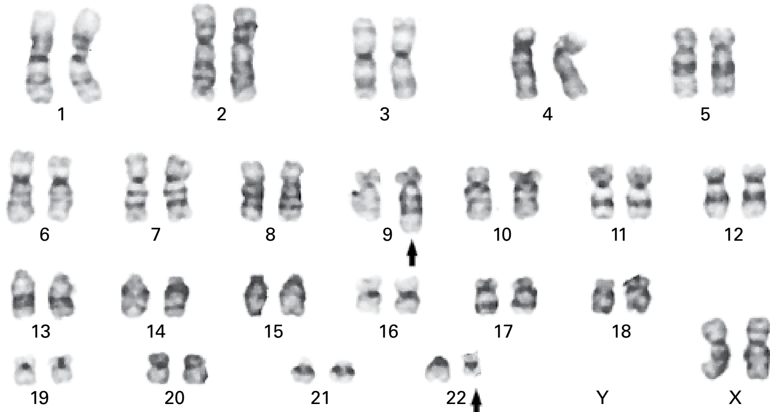


Fig. 6.4 Karyogram of a patient with CML showing t(9;22)(q34.1;q11.2). The 22q− derivative chromosome is the Ph chromosome. (With thanks to Professor Lorna Secker-Walker.)

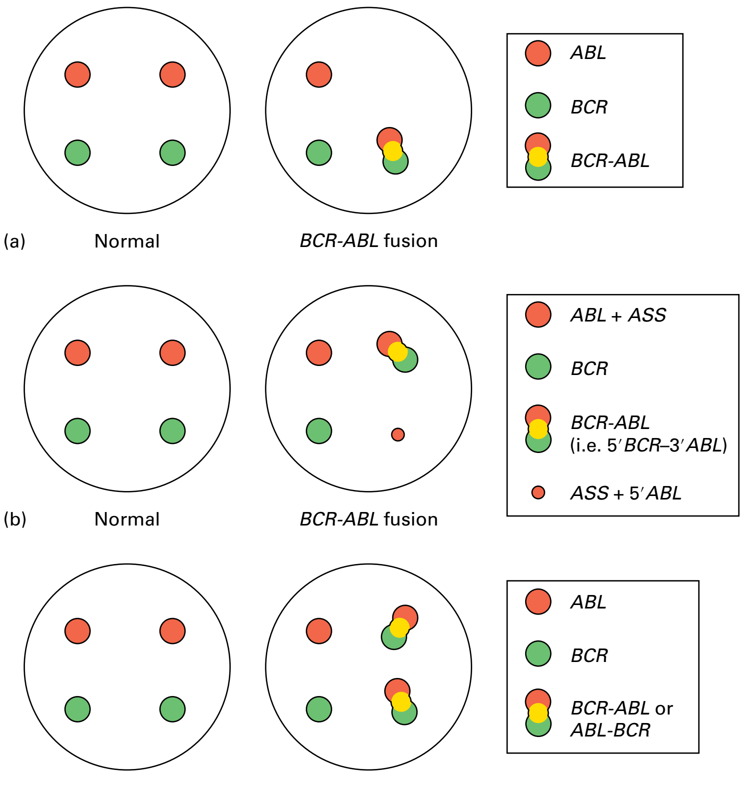


Fig. 6.5 Diagrammatic representation of three fluorescence *in situ* hybridization (FISH) strategies for detection of *BCR-ABL1* fusion. (a) Dual-colour, single-fusion FISH. Normal cells have two red *ABL1* signals and two green *BCR* signals. When t(9;22) is present, there is a red *ABL1* signal, a green *BCR* signal and a yellow *BCR-ABL1* fusion signal. (b) Extra-signal, dual-colour FISH. The *ABL1* probe encompasses also the upstream *ASS* 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.

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 [36]. RT-PCR is more sensitive than FISH and is made quantitative in the RQ-PCR technique, in which transcripts are compared to those of another gene, such as *ABL1*. Neither FISH, RT-PCR nor RQ-PCR permits the detection of secondary cytogenetic abnormalities in the Ph-positive clone or 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.

A concomitant *JAK2* V617F mutation is found in a low proportion of patients with CML, in one

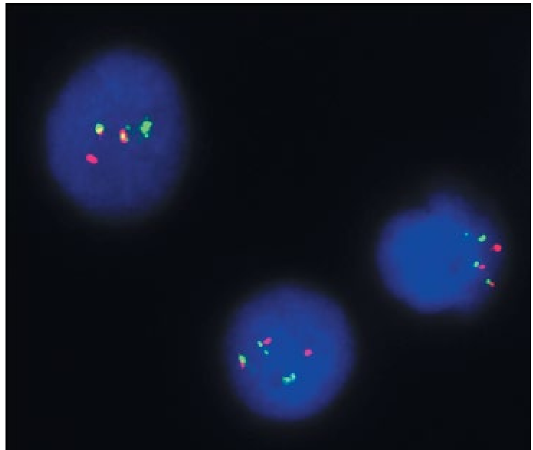


Fig. 6.6 Dual-colour, dual-fusion FISH in CML. The green probe is for *BCR* on chromosome 22, whilst the red probe (actually Spectrum Orange) is for *ABL1* on chromosome 9. Both probes span their respective breakpoints. In the nucleus of a normal cell, there would be two red and two green signals. In 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). (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

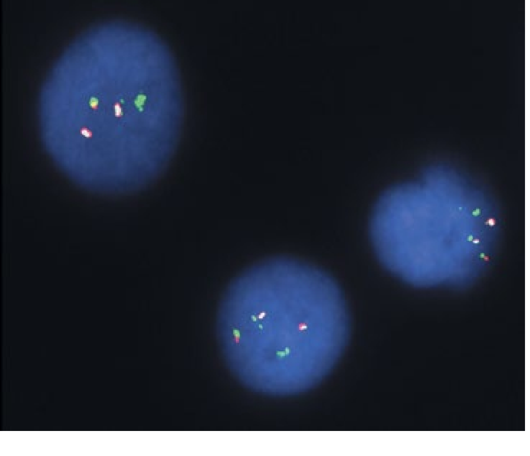


Fig. 6.7 Dual-fusion, tricolour FISH probe in CML with a supplementary aqua probe (appears pale blue or white) for *ASS*, which is proximal to *ABL1*. The same cells as in Fig. 6.6 have been studied and the *ASS* probe results have been overlayed on the image. The results are those expected with a standard 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 on the derivative chromosome 9 and a red–yellow–green fusion signal on the derivative chromosome 22. No signals have been lost. (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology.)

series in 2.55% of 314 patients on treatment for chronic phase disease [37]. Chronic myeloid leukaemia may develop more frequently than would be expected by chance in patients with *JAK2* V617F-positive MPN [38]. Coexistence with *JAK2* V627F-positive polycythaemia vera, essential thrombocythaemia and primary myelofibrosis has been observed [38,39]. In two such patients, the *BCR-ABL1* mutation had occurred in a stem cell that was *JAK2* V617F positive [40]. *JAK2* V617F can also occur in an independent clone in *BCR-ABL1*-positive CML [37], with dominance of the mutant *JAK2* clone leading to megakaryocytes with 'staghorn' nuclei replacing the relatively small hypolobated megakaryocytes of CML [41]. Rarely *BCR-ABL1* and a *CALR* mutation are found in cells of a single clone [42]. CML has also developed as a second event in a patient with essential thrombocythaemia as a result of the *BCR-ABL1* mutation occurring in a cell of a *CALR*-mutated clone [43]. Occasional patients with the haematological features of MDS have been found to have either t(9;22) or *BCR-ABL1* fusion, sometimes associated with a complex karyotype [44]; it is likely that this represents clonal evolution before the development of the clinical and haematological features of chronic phase CML.

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 [45,46], 6% of 258 in a third [47], and such changes in as many as

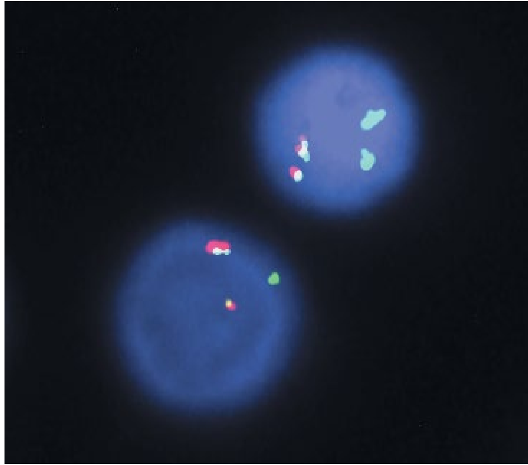


Fig. 6.8 Dual-fusion, tricolour FISH probe in CML with a supplementary aqua probe (appears pale blue or white) for *ASS*, which is proximal to *ABL1*. The technique is the same as in Fig. 6.7 but illustrates a deletional variant. There is a normal cell (top right) with two green *BCR* signals and two 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. (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology.)

24% of patients achieving a complete or partial cytogenetic remission in a fourth series [48]. The abnormalities observed included those that occur in secondary leukaemias such as del(5q), −7, del(7q), del(13q), del(20q), +8, +11, −X and −Y [45,46,48–50]. Clonally independent molecular abnormalities can also occur, including *NRAS* mutation [51] and *JAK2* V617F [52]. *WT1* overexpression can be a marker of a Ph-negative, cytogenetically aberrant clone [50]. These clonal abnormalities are not generally accompanied by myelodysplastic features and occasionally they disappear [48]. However, they may be the harbinger of development of Ph-negative AML, high grade MDS [6,47,53] or a Ph-negative MPN or MDS/MPN [51,52]. Acute leukaemia associated with inv(16) originating in Ph-negative cells has also been reported [54]. Patients treated with dasatinib can similarly develop clonal cytogenetic abnormalities in Ph-negative cells [55].

Chronic myeloid leukaemia in accelerated phase and blast transformation

After a variable period in chronic phase, usually several years, CML 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 [56]. In the 2016 revision of the WHO classification the following criteria for accelerated phase are recognized: (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) persistent or increasing WBC ($>10 \times 10^9$/l), or persistent or increasing spleen size that does not respond to treatment; (v) presence at diagnosis of a second Ph, trisomy 8, isochromosome 17q, trisomy 19, a complex karyotype or abnormalities of 3q26.2; (vi) cytogenetic evolution; or (vi) prominent proliferation of small dysplastic megakaryocytes in large clusters or sheets, associated with reticulin and collagen fibrosis (presumptive evidence) [4]. In addition further criteria relating to response to treatment have been accepted: (i) haematological resistance to first-line TKI therapy; (ii) any grade of resistance to two subsequent TKIs; or (iii) occurrence of two or more *BCR-ABL1* mutations during therapy [4]. Whether cytogenetic evolution alone should be regarded as indicative of accelerated disease has previously 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 [5].

Blast transformation may be myeloid, lymphoid or mixed phenotype. 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 [57]. Lymphoid blast crisis is usually B lineage but can be T lineage [58] and rarely is NK lineage [59]. B-lineage lymphoid blast crisis may be early precursor B cell or have a common ALL or a pre-B phenotype. In lymphoid blast crisis there may be coexpression of myeloid antigens. Mixed phenotype leukaemia is relatively much more common in CML in transformation than in *de novo* acute leukaemia. 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 (presumptive evidence) [4].

Clinical and haematological features

During the accelerated phase of CML 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. Monocytosis is occasionally seen [60]. 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, skin infiltration 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. 6.9). A minority of patients have hypogranular neutrophils or the acquired Pelger–Huët anomaly [61]. Blast transformation may be myeloid, lymphoid or mixed phenotype. Myeloid transformation may be multilineage or may be predominantly myeloblastic, monoblastic, myelomonocytic, hypergranular promyelocytic, eosinophilic, basophilic and/or mast cell, megakaryoblastic or erythroid. Mixed phenotype transformation can be B-myeloid or T-myeloid. In pure lymphoid blast

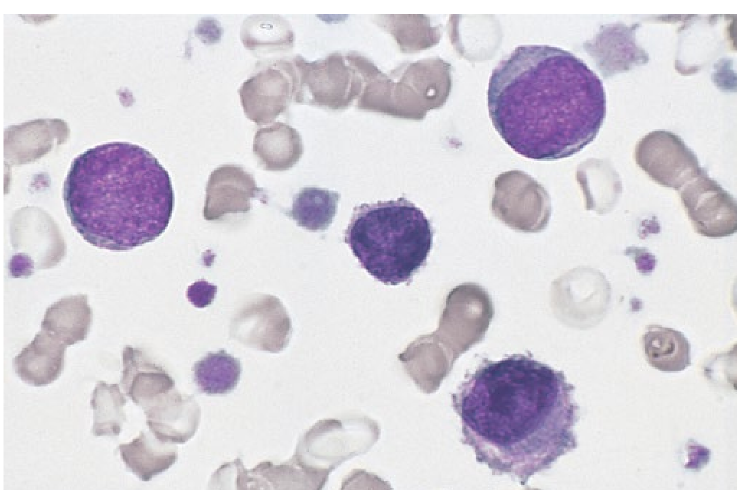


Fig. 6.9 PB film of a patient with megakaryoblastic transformation of CML showing large platelets, several blast cells and a micromegakaryocyte. MGG ×100.

crisis there are 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 French–American–British (FAB) categories of L1 or L2 ALL but rarely they resemble the blasts of L3 ALL [62].

Since the blast cells of acute transformation often show no cytological evidence of differentiation, immunophenotyping may be necessary to confirm their lineage.

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 [57].

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 specific 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%) [23–25]. It should be noted that the secondary cytogenetic abnormality usually described as i(17q) may, in fact, be idic(17p11.2) [24]. 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.* [24] 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;q22.1), t(15;17)(q24.1;q12), inv(16)(p13.1q22), chromosomal rearrangements with a 3q21.3 or 3q26.2 breakpoint – t(3;21)(q26.2;q22.1), t(3;3)(q21.3;q26.2) and inv(3)(q21.3q26.2) [24] – and t(3;11)(q21;q23), t(9;11)(p22;q23), t(11;17)(q23;q21) and t(11;19)(q23;p13.3) with rearrangement of *KMT2A* [63]. Chromosome 3q21.3 and 3q26.2 abnormalities are often associated with large numbers of lymphocyte-sized micromegakaryocytes [64], whereas i(17q) or idic(17p11.2) is associated with Pelger–Huët neutrophils and prominent peripheral blood basophilia, the basophil count being usually more than 10% and often more than 20% [24,65]. The appearance of 3q26.2 rearrangement including inv(3), t(3;3) and t(3;21) during chronic or accelerated phase disease is associated with rapid transformation to blast phase and resistance to TKIs [66].

Acquisition of t(15;17) can be associated with cytological features resembling acute promyelocytic leukaemia, and of inv(16) with features resembling M4Eo AML [67]. Recognition of acute promyelocytic transformation may be important because of its responsiveness to all-*trans*-retinoic acid (ATRA) [68]. Monoblastic transformation can occur when there is a rearrangement with an 11q23.3 breakpoint [69].

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 [70].

In TKI-treated patients analysed at all stages of the disease, −Y was found to be of no prognostic significance in comparison with patients with no additional cytogenetic abnormality; +8 or + Ph was associated with a worse prognosis in the presence of i(17)(q10), −7/7q−, 3q26.2 rearrangement or the presence of two or more additional cytogenetic abnormalities [71]. However, +8 and +Ph had no adverse significance when they appeared during chronic phase disease [71].

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* [23,72–75]. Mutation of *RUNX1* is observed in about 13% of patients with disease in acute transformation [76]. *ASXL1* may be mutated in myeloid blast crisis. Amplification of *BCR-ABL1* has been associated with refractoriness to imatinib therapy [74]. 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 [23,72,73]. Homozygous deletions of *CDKN2A*, encoding $p16^{INK4A}$, are associated with lymphoid blast crises, being seen in about 50% of cases [23,77]. *IKZF1* may be mutated in lymphoid blast crisis.

Problems and pitfalls

Beware of missing t(9;22)(q34.1;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 (CNL) is a rare Ph-negative condition that, 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 [78]. It appears likely that some cases result from mutation in a multipotent stem cell and others from a mutation in a committed granulocyte precursor [79]. Familial cases have been described [80] but since a *CSF3R* mutation has been described in a family with hereditary neutrophilia it is important to exclude a constitutional abnormality in such cases. Some cases have followed cytotoxic chemotherapy [81] and are classified as therapy-related myeloid neoplasms.

A similar disease phenotype is sometimes seen as an accelerated phase of an MPN, for example supervening in polycythaemia vera [82], but such cases are not classified as CNL.

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 [83]. It is more common in men than in women [84]. Characteristic clinical features are fatigue, weight loss, fever, sweats, splenomegaly and sometimes hepatomegaly [81]; pruritus and gout can also occur, and mucosal bleeding is not uncommon. Many patients, however, are asymptomatic [81]. The reported median survival has ranged from little more than a year [83] to 2–3 years [79,81].

The neutrophil count is markedly increased but the peripheral blood shows few granulocyte

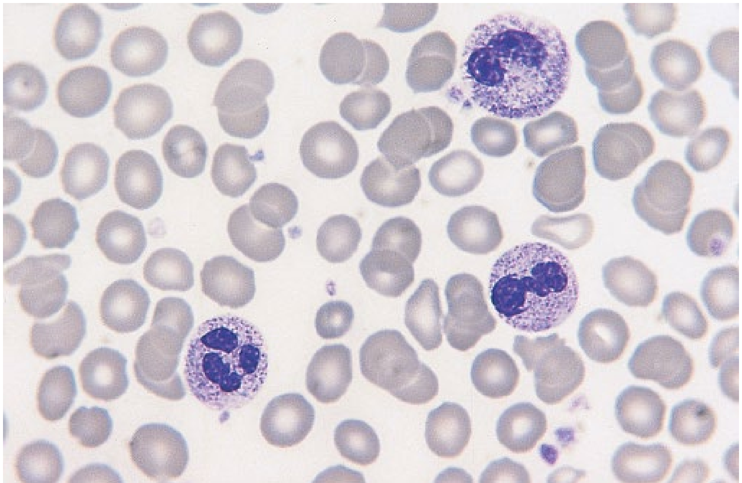


Fig. 6.10 PB film of a patient with neutrophilic leukaemia showing an increase of mature neutrophils, which show toxic granulation. MGG ×100.

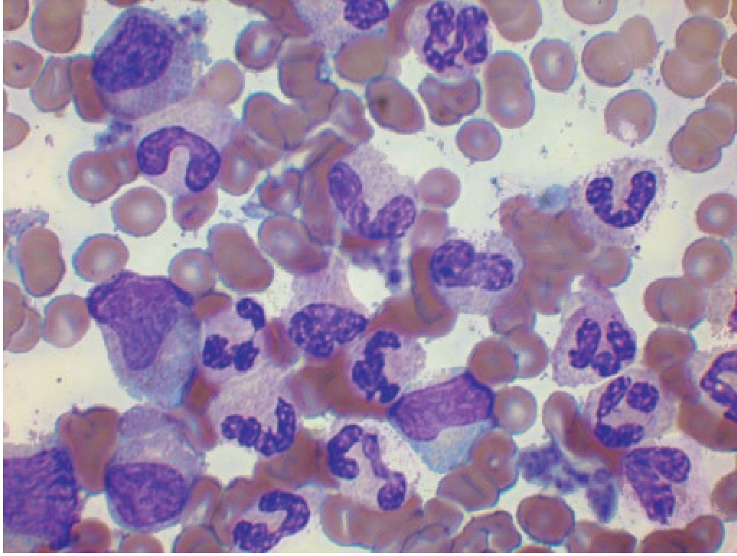


Fig. 6.11 BM film from a patient with chronic neutrophilic leukaemia showing an increase of cells of neutrophil lineage with morphologically normal maturation. MGG ×100.

precursors (Fig. 6.10). Toxic granulation and Döhle bodies are often present, and ring-shaped neutrophil nuclei have also been described [84]. 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 [85], but in the WHO classification such cases would not be classified as MDS/MPN, unclassifiable rather than as CNL. The NAP score is usually high but may be reduced and is not diagnostically useful. Serum vitamin B_{12} concentration is increased. There may be hyperuricaemia, and gout has occurred. Serum granulocyte colony-stimulating factor (G-CSF) is reduced. The bone marrow (Fig. 6.11) is markedly hypercellular with a minor degree of left shift [83]. Some cases have developed bone marrow fibrosis and osteosclerosis [86]; an alternative explanation of such cases is that the initial presentation represented the hypercellular phase of primary myelofibrosis.

In the 2016 revision of the WHO classification, the criteria for the diagnosis of CNL are: (i) WBC at least 25×10^9/l, neutrophils and band forms at least 80% of white cells, blast cells rarely observed, immature granulocytes (metamyelocytes to promyelocytes) less than 10% of white cells, monocytes less than 1.0×10^9/l and no neutrophil dysplasia; (ii) bone marrow showing increased neutrophils and precursors with normal maturation and blast cells less than 5% of nucleated cells; (iii) not meeting WHO criteria for any other myeloid neoplasm, specifically no *BCR-ABL1*, no rearrangement of *PDGFRA*, *PDGFRB* or *FGFR1* and no *PCM1-JAK2*; and (iv) presence of *CSF3R* T618I or other activating *CSF3R* mutation or, in its absence, neutrophilia persisting for at least 3 months, splenomegaly and no identifiable cause of reactive neutrophilia including absence of a plasma cell neoplasm or, if present, demonstration of clonality of myeloid cells by cytogenetic or molecular studies [78].

It is important to distinguish CNL 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 polyclonal [79,84,87]. However, it should be noted that two of five patients investigated for plasma cell neoplasia-associated neutrophilia were found to have a *SETBP1* mutation (but not a *CSF3R* mutation) suggesting that there are sometimes two coexisting clonal disorders [88].

Blastic transformation of chronic neutrophilic leukaemia has occurred in a fifth of reported cases [79,89,90]. 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. In other patients, the disease terminates with a rising white cell count that is refractory to chemotherapy [91]. With disease evolution, haematological features may resemble those of chronic myelomonocytic leukaemia (CMML).

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, monosomy 7, del(11q), del(12p), del(20q) and several non-recurrent translocations [79,89,90] but the karyotype is more often normal [24,79]. An activating mutation in the proximal membrane domain of *CSFR3* (encoding the receptor for G-CSF) was found in eight of nine patients [92] and is now regarded as typical of CNL [78]; in five of these patients there was also a mutation in *SETBP1* [93]. *ASXL1* mutation is a common coexisting abnormality and correlates with worse survival [81]. The only other condition in which *CSFR3* mutation has been frequently reported is aCML, mutation being found in 7 of 18 cases [91]; however, this observation was not confirmed in subsequent studies [94,95]. The *JAK2* V617F mutation has been reported in more than a dozen patients [84,96,97] although in one of these patients a diagnosis of essential thrombocythaemia may have been more appropriate [97]. 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 [98]. In those with an initially normal karyotype, a clonal cytogenetic abnormality may appear during the course of the disease.

Identification of *CSF3R* mutations may be clinically important since a response to ruxolitinib can be seen [93].

Problems and pitfalls

Beware of making a diagnosis of chronic neutrophilic leukaemia in patients with plasma cell neoplasms [84]. The presence of toxic granulation, Döhle bodies or an elevated NAP do not help in making a distinction [84,99]. Serum protein electrophoresis is indicated in all patients who lack definitive evidence of a myeloid neoplasm. However, it should be noted that occasionally a plasma cell neoplasm coexists with chronic neutrophilic leukaemia [88,100]. The distinction between CNL and aCML can

sometimes be difficult; it may be that cases with a *CSF3R* mutation and monocytosis represent disease evolution in CNL.

Chronic eosinophilic leukaemia, not otherwise specified and related conditions

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;q22.1), that identifies them as AML. In the 2016 revision of the WHO classification, cases of eosinophilic leukaemia characterized by rearrangement of one of three tyrosine kinase genes (*PDGFRA*, *PDGFRB* or *FGFR1*) or with *PCM1-JAK2, ETV6-JAK2* or *BCR-JAK2* are classified according to the molecular abnormality (see below) and are thus excluded from the category of chronic eosinophilic leukaemia, not otherwise specified (CEL, NOS) (Table 6.3) [2,3]. Clinical and pathological features are similar in these different groups but there are some disease characteristics associated more specifically with specific syndromes (see below). It should be noted that grouping myeloid neoplasms according to the molecular abnormality is more biologically meaningful than grouping them according to the presence or absence of eosinophilia.

Clinical and haematological features

Patients with chronic 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.

Eosinophils are increased in the blood and there may also be an increase of eosinophil precursors including blast cells (Fig. 6.12). An eosinophil count of at least 1.5×10^9/l is a WHO

Table 6.3 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, rarely B lymphoid transformation
Myeloid and lymphoid neoplasms associated with *PDGFRB* rearrangement	CEL, CMML with eosinophilia, aCML with eosinophilia; bone marrow mast cells may be increased; may undergo myeloid transformation; T and unspecified lymphoid transformations have been reported
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
Myeloid and lymphoid neoplasms with *PCM1-JAK2*	Heterogeneous, including CEL, aCML and B lymphoblastic transformation

aCML, atypical chronic myeloid leukaemia (*BCR-ABL1* negative); ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CMML, chronic myelomonocytic leukaemia.

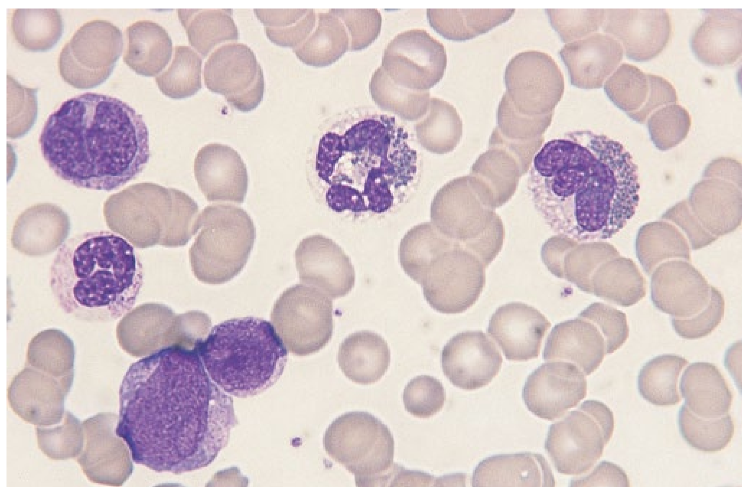


Fig. 6.12 PB film of a patient with chronic eosinophilic leukaemia with trisomy 10 showing a neutrophil, two abnormal eosinophils, a granulocyte precursor and two blast cells. MGG ×100.

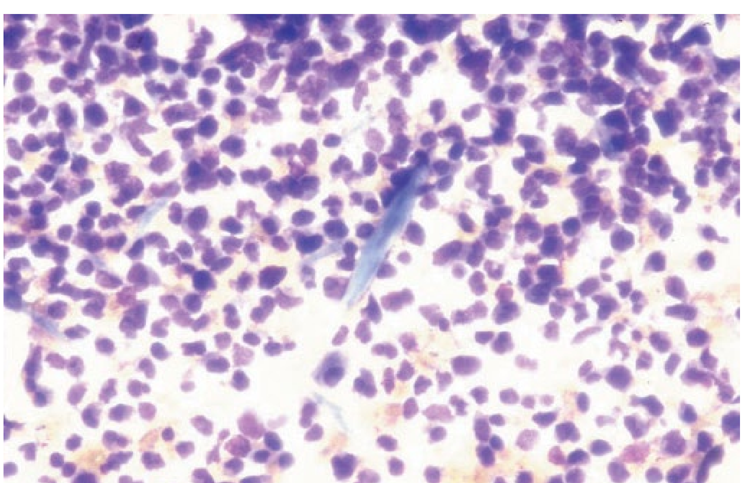


Fig. 6.13 BM film from a patient with eosinophilic leukaemia showing a Charcot–Leyden crystal. MGG ×40.

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 [101]; the same patient had periodic acid–Schiff-positive cytoplasm [101]. One patient has been described in whom there was cycling of the WBC, Hb and platelet count [102].

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. 6.13). Trephine biopsy sections show the same features.

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 (idiopathic HES). The nature of the latter condition is, by definition, not known. 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 can 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 [103]. 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 [104].

Prognosis of chronic eosinophilic leukaemia, not otherwise specified, is poor with acute transformation being common [105]. This is usually to AML, but one instance of transformation to T lymphoblastic leukaemia/lymphoma was reported [105].

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)(q32;p13.2) and other translocations with *PDGFRB* rearrangement; (ii) *FIP1L1-PDGFRA* fusion and other rearrangements of *PDGFRA*; (iii) t(8;13)(p11.2;q12.1) and other rearrangements of *FGFR1*; and (iv) cases with *PCM1-JAK2*, *BCR-JAK2* or *ETV6-JAK2* (a provisional category). These groups of disorders are excluded from the category of CEL, NOS, and are discussed below.

A number of clonal cytogenetic abnormalities have been observed including monosomy 7, trisomy 8, i(17q), trisomy 15, del(20)(q11q12) and the presence of trisomy 8 and trisomy 21 in different clones. One patient has been reported with a complex karyotype including t(2;4)(p13;p15) [105]. Atypical CML with marked eosinophilia can result from t(5;12)(q31.1;p13.2) with *ETV6-ACSL6* (*ACS2*), and overexpression of interleukin 3 [106]. A patient with CEL with an *ETV6-ABL1* fusion gene associated with a complex chromosomal rearrangement involving chromosomes 9, 12 and 17 has been reported [107]; the same fusion gene resulting from t(9;12)(q34.1;p13.2) has been associated also with MPN with eosinophilia [108], with AML with eosinophilia and with aCML without eosinophilia [109]. Disease associated with *ETV6-ABL1* is sensitive to imatinib and nilotinib [110]. CEL can result from t(12;13)(p13.2;q12.2) generating an *ETV6-FLT3* fusion gene [111,112]; one (and probably both) of the patients reported by Walz *et al.* had an associated T lymphoblastic lymphoma [112]. *ETV6-FLT3* has also been associated with CEL with evolution to mixed phenotype T-lymphoid/myeloid acute leukaemia in lymph nodes [113] and with primary myelofibrosis with eosinophilia [114]. Disease associated with *ETV6-FLT3* may be sensitive to sunitinib and sorafenib [112,114]. Another patient with an MPN with eosinophilia had *ETV6-SYK* fusion associated with t(9;12)(q22.2;p13.2) [115]. *JAK2* V617F has been reported occasionally, with transformation to AML occurring in one patient [116]; there may be associated cardiac damage or Budd–Chiari syndrome [116–118]. A *KIT* M541L mutation was reported in four patients, the disease being imatinib responsive [119]. Occasional patients have a *CBL* mutation [120] or a loss-of-function *EZH2* mutation [121].

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) [122].

By means of targeted next generation sequencing it is possible to identify clonal genetic abnormalities in more than a quarter of patients who would otherwise be classified as idiopathic HES; genes implicated include *ASXL1*, *TET2*, *EZH2*, *SETBP1*, *CBL* and *NOTCH1* [123]. Categorization of such patients as CEL, NOS may be appropriate.

Problems and pitfalls

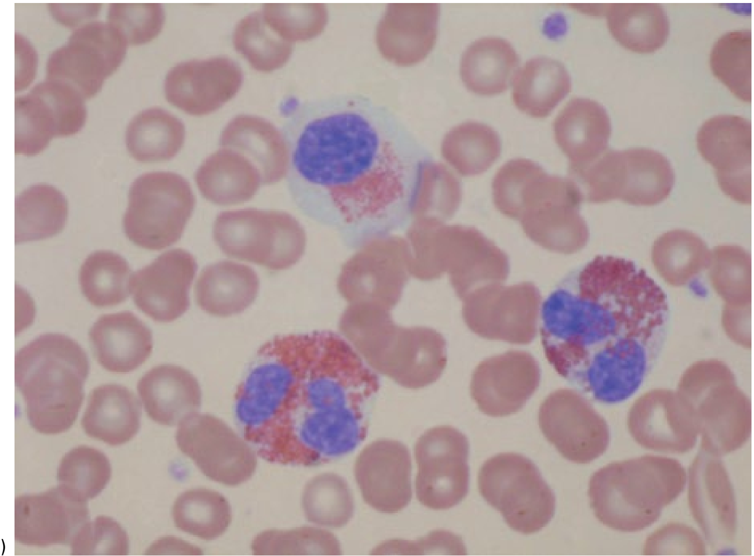
It is important to be aware that there may be some patients with CEL whose disease cannot be recognized at presentation because they do not have an increase of blast cells, a clonal cytogenetic abnormality or a known molecular genetic abnormality.

Myeloid and lymphoid neoplasms associated with *FIP1L1-PDGFRA* or other rearrangement or activating mutation of *PDGFRA*

The discovery of a recurring molecular genetic abnormality in a group of patients previously considered to have idiopathic HES 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 [104,124]; subsequently this entity was incorporated into the WHO classification [2]. It is recognized that T lymphoblastic and myeloid transformations occur and that in some patients the disease presents as acute leukaemia [125]. B lymphoblastic transformation can also occur [126]. The causative mutation arises in a pluripotent stem cell able to give rise to eosinophils, neutrophils, monocytes, B lymphocytes, T lymphocytes and mast cells [127] but, in chronic phase disease, abnormal proliferation is largely confined to eosinophils, neutrophils and mast cells. Because of its responsiveness to imatinib (see below), 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. Cough is a prominent symptom [128]. A significant proportion of patients are asymptomatic, diagnosis being incidental [128]. Clinical associations include lymphomatoid papulosis [129] and mucosal ulceration. Lytic bone lesions are an uncommon manifestation [130]. There may be mild anaemia and thrombocytopenia, a moderately elevated WBC and a moderately to markedly elevated eosinophil count (Fig. 6.14). Reported series of cases have included patients with 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 [131]. Rare patients have a chronic myeloid leukaemia without eosinophilia [132–134]; in one such patient the haematological features simulated CNL [134]. Presentations with myeloid sarcoma with eosinophilia [135] and with monoblastic sarcoma with bone marrow but not peripheral blood eosinophilia [136] have been described. The neutrophil count may be increased and some circulating granulocyte precursors may be present. Thrombocytosis is occasionally observed [137]. Serum vitamin B_{12} is markedly elevated. Serum tryptase is elevated but generally to a lesser extent than is seen in systemic mastocytosis [124,128]. Serum immunoglobulin E is elevated in a minority of patients [128,131]. The bone marrow is hypercellular with increased eosinophils and precursors (Fig. 6.15). Trephine biopsy sections show an increase of eosinophils and precursors (Fig. 6.16) and, in addition, mast cells are often increased and may be atypical, for example spindle shaped; usually they form loose clusters [124] but sometimes there are cohesive aggregates [138] or sheets, the features thus resembling systemic mastocytosis. The mast cells, like those of systemic mastocytosis, often show aberrant expression of CD25 but they differ in that CD2 is


(a)

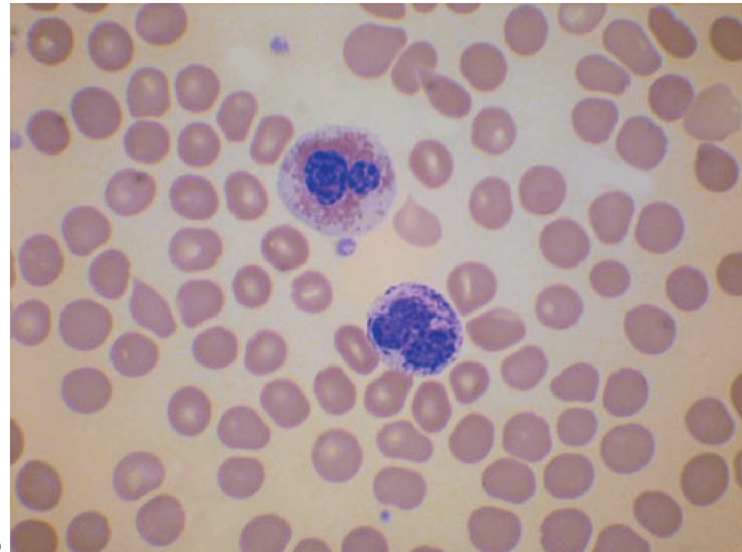

(b)

Fig. 6.14 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.

not usually aberrantly expressed [124]. Reticulin deposition is often increased.

Transformation to acute leukaemia can occur after a variable period of time [125]. 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 [125] and rarely, in the case of *BCR-PDGFRA* fusion, B lymphoblastic leukaemia [126]. Presentation as T lymphoblastic lymphoma (with associated myeloid neoplasm and eosinophilia) has also been reported [108].

Disease due to *FIP1L1-PDGFRA* is very sensitive to imatinib; not only patients presenting with CEL but even those who present with AML [136,139], including myeloid sarcoma [135,140],

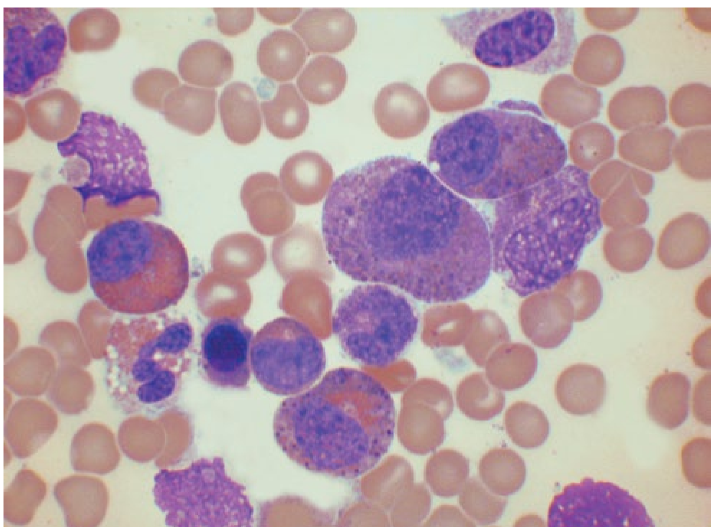
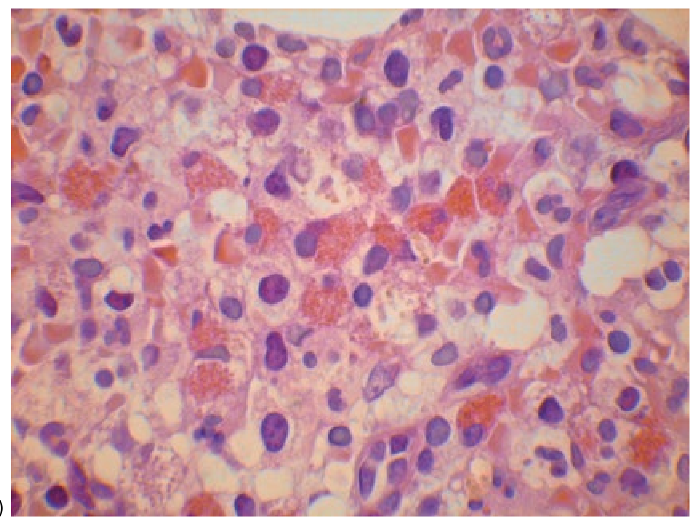
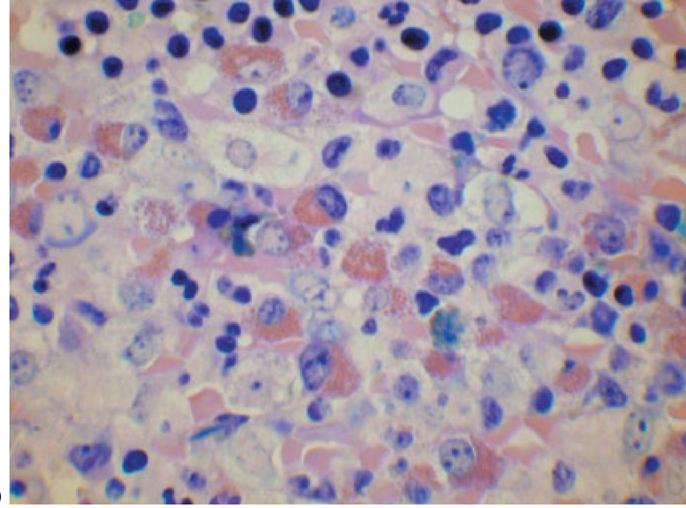


Fig. 6.15 BM film in a patient with *FIP1L1-PDGFRA* showing eosinophil precursors, some of which have pro-eosinophilic granules with basophilic staining characteristics; there is also a vacuolated, partly degranulated, mature eosinophil. MGG ×100.



(a)



(b)

Fig. 6.16 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.

may respond. In one study of seven patients treated with imatinib alone in blast phase, all had a complete remission with only two patients relapsing [141]. Rarely institution of imatinib therapy has been followed by the development of reversible pure red cell aplasia [142]. Occasional patients have been reported in whom molecular remission was maintained on cessation of imatinib therapy [128].

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 [104] or by other rearrangement or an activating mutation [143] of *PDGFRA* (Table 6.4) [2,108,143–148]. In patients with *FIP1L1-PDGFRA* fusion, cytogenetic analysis may be normal or show a clonal cytogenetic

Table 6.4 Chronic eosinophilic leukaemia and related conditions associated with rearrangement of *PDGFRA* [2,108,143–148]; the most common rearrangement is shown in bold.

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-ALL with eosinophilia**
t(2;4)(p22.2;q12)	*STRN-PDGFRA*	2	CEL; AML without eosinophilia [108]
t(4;10)(q12;q23.3)	*TNKS2-PDGFRA*	1	CEL [144]; AML without eosinophilia [108]
t(4;12)(q12;p13.2)	*ETV6-PDGFRA*	3	CEL, marked basophilia in one patient* [145]
Complex karyotype including t(4;14)(q12;q24)	*PDGFRA* rearrangement postulated	1	t-AML with eosinophilia and basophilia [146]
t(4;22)(q12;q11.2)	*BCR-PDGFRA*	At least 9	Intermediate features between CEL and Ph-positive CML; T and B lymphoid transformation can occur; T-ALL with eosinophilia [147] and mixed phenotype (B lymphoid-myeloid) acute leukaemia without eosinophilia have been reported [148]
ins(9;4)(q33;q12q25)	*CDK5RAP2-PDGFRA*	1	CEL
Complex involving chromosomes 3, 4, 10 and probably 13	*KIF5B-PDGFRA* (*KIF5B* is located at 10p12-cen)	1	CEL†
Point mutation in *PDGFRA*	Point mutation in *PDGFRA*	Four mutations in three patients	CEL [143]

AML, acute myeloid leukaemia; CEL, chronic eosinophilic leukaemia; CML, chronic myeloid leukaemia; T-ALL, T acute lymphoblastic leukaemia; t-AML, therapy-related AML.

* One patient imatinib sensitive, one resistant.

† Imatinib sensitive.

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) [104] or t(4;10)(q12;p11) [149]. Unrelated abnormalities have included trisomy 8 [150] and a complex karyotype including trisomy 8 and trisomy 19 [104]. Additional cytogenetic abnormalities, such as trisomy 8 and trisomy 9, may be present at evolution to AML [125]. 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 with relapse of disease or transformation to AML [151,152]. The T674I mutation leads to loss of sensitivity to imatinib; nilotinib and sorafenib appear effective *in vitro* but are clinically not very effective. Midostaurin may possibly be of benefit. A single patient has been reported with imatinib resistance at presentation due to tandem S601P and L629P mutations in the kinase domain of *PDGFRA* within the fusion gene [153]. The *FIP1L1-PDGFRA* fusion gene has also been detected at the time of transformation of CMML to AML with eosinophilia [154].

Microdissection of mast cells has demonstrated that a proportion of patients have a low-level D816V *KIT* mutation in mast cells, and in one patient FISH provided evidence of *FIP1L1-PDGFRA* in mast cells [155].

Variant translocations associated with rearrangement of *PDGFRA* and a different partner gene are summarized in Table 6.4. 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 Ph-positive CML.

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. 6.17) are recommended. Because of the potential for cardiac damage, delay in diagnosis should also be

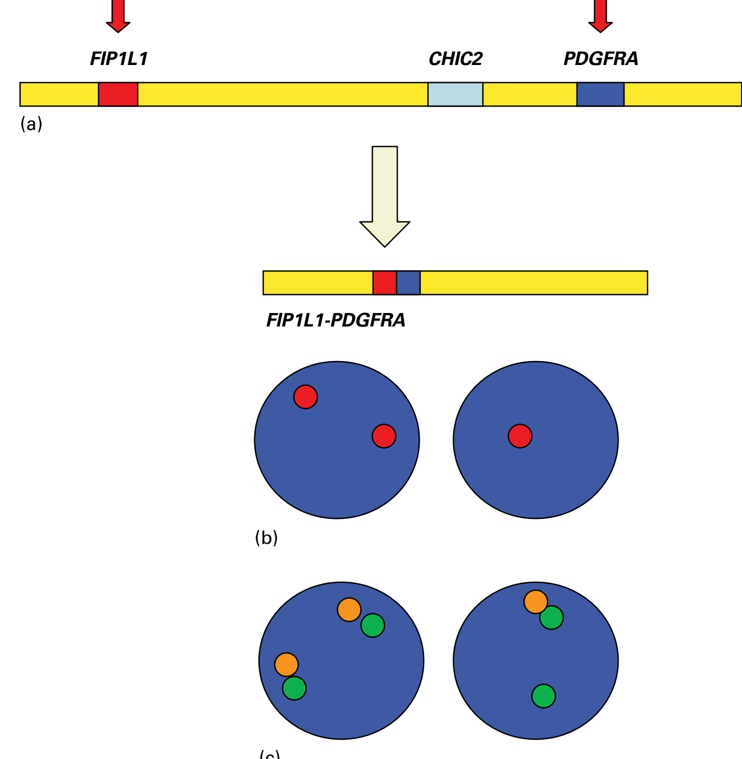


Fig. 6.17 Diagrams illustrating the loss of *CHIC2* when a *FIP1L1-PDGFRA* fusion gene is formed as a result of a cryptic deletion at 4q12. (a) Diagram showing the occurrence of an interstitial deletion with loss of *CHIC2*; the red arrows indicate the two breakpoints. (b) A FISH technique using a *CHIC2* 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.

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 TKIs, even when they present with the disease in the acute phase.

Myeloid and lymphoid neoplasms associated with rearrangement of *PDGFRB*

Myeloid neoplasms associated with rearrangement of *PDGFRB* form a somewhat heterogeneous group [2]. The majority of patients have a chronic myeloid neoplasm with prominent eosinophilia, t(5;12)(q32;p13.2) and *ETV6-PDGFRB* but eosinophilia may be absent. However, only about half of patients with this translocation are found to have *ETV6-PDGFRB*, and those that do not are excluded from the category. At least 24 other fusion partners have been reported (Table 6.5) [2,103,108,156–180]. The myeloid neoplasm most often has the haematological features of CMML, aCML with eosinophilia, or CEL, but cases have also been reported of AML with eosinophilia, of primary myelofibrosis transforming to AML [159], of primary myelofibrosis with eosinophilia [179] and of MDS with eosinophilia [179]. The condition can occur in infants and children, and one case of congenital JMML with eosinophilia has been reported [160]. Rarely there is a lymphoid component, either T lymphoblastic [108,170,171] or unspecified [181]. Patients may present in myeloid or lymphoid blast crisis [181]. Neoplasms with a *PDGFRB* fusion gene are imatinib responsive. Even patients presenting in T lymphoblastic or myeloid blast crisis may have a sustained response [181].

In addition to the predominantly myeloid neoplasms tabulated, certain specific *PDGFRB* fusion genes, for example *EBF1-PDGFRB* [182], *ATF7IP-PDGFRB* [183], *SSBP2-PDGFRB*, *TNIP1-PDGFRB* and *ZEB2-PDGFRB*, have been associated with precursor B-ALL (usually with a Ph-like molecular signature) without eosinophilia and are categorized separately (see page 267).

Chronic myelomonocytic leukaemia with eosinophilia (eosinophil count 1.4×10^9/l) associated with t(5;12)(q33;p13.2) and *ETV6-PDGFRB* has been reported as an apparent therapy-related leukaemia 9 years after treatment of acute promyelocytic leukaemia [184].

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 (including a 'blueberry muffin' appearance in a congenital case) [160]. The haematological features are variable (see above). In addition to the frequent occurrence of eosinophilia (Figs 6.18 and 6.19), some patients have had neutrophilia, monocytosis, basophilia or circulating granulocyte precursors. There may be anaemia, thrombocytopenia and multilineage myelodysplasia. Although eosinophilia is very common, it does not appear to be invariable. In two patients with t(4;5)(q21.2;q32) or a variant translocation and a *PRKG2-PDGFRB* fusion gene, the haematological features were those of chronic basophilic leukaemia [164,165]. Bone marrow mast cells may be increased, may be spindle-shaped and may show aberrant expression of CD2 and CD25; serum tryptase may be increased [164,165]. Serum vitamin B_{12} concentration is often increased [131]. Pre-imatinib, acute transformation occurred in 16% of reported patients and the median survival was less than 2 years [157]. A small series of patients suggests that with imatinib median survival is of the order of 5 years [185].

Cytogenetic and molecular genetic features

This condition is defined by the presence of rearrangement of *PDGFRB* in the context of a

Table 6.5 Chronic eosinophilic leukaemia and related conditions associated with rearrangement of *PDGFRB* [2,103,108,156–180]; the most common rearrangement is shown in bold.

Chromosomal rearrangement	Fusion gene	Haematological presentation
t(5;12)(q32;p13.2) 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** [2,103,108,156–159]
t(1;3;5)(p36;p22.3;q32)	*WDR48-PDGFRB* (*WDR48* is at 3p22)	CEL
t(1;5)(q21.3;q32)	*TPM3-PDGFRB*	CEL, JMML with eosinophilia (fusion gene not sought) [160]
t(1;5)(q21.2;q32)	*PDE4DIP-PDGFRB*	MDS/MPN with eosinophilia
t(2;5)(p16.2;q32)	*SPTBN1-PDGFRB*	MPN with eosinophilia; MDS evolving to AML [161]
t(3;5)(p22.2;q32)	*GOLGA4-PDGFRB*	CEL or aCML with eosinophilia (two cases) [162]
t(3;5)(q13.3;q32)	*GOLGB1-PDGFRB*	CEL [163]
t(4;5;5)(21.2;q31;q32) or t(4;5)(q21.2;q32)	*PRKG2-PDGFRB*	Two cases with chronic basophilic leukaemia and abnormal bone marrow mast cells [164,165], one case with MPN with abnormal mast cells and an eosinophil count of 1.1×10^9/l [166]
Cryptic interstitial deletion of 5q	*TNIP1-PDGFRB*	CEL with thrombocytosis and increased mast cells [167]; MPN with eosinophilia [168];
t(5;6)(q32;q22.3)	*CEP85L-PDGFRB* (*C6orf204-PDFGRB*)	One case of CEL [169] and two cases of MPN with eosinophilia and T-LBL [108,170,171]
t(5;7)(q32;q11.2)	*HIP1-PDGFRB*	CMML with eosinophilia
t(5;7)(q32;p13-14.1)	*HECW1-PDGFRB* (*NEDL1-PDGFRB*)	JMML [172]
t(5;9)(q32;p24.3)	*KANK1-PDGFRB*	Essential thrombocythaemia without eosinophilia [173]
t(5;10)(q32;q21.2)	*CCDC6-PDGFRB*	MPN with eosinophilia or aCML
der(1)t(1;5)(p34;q32), der(5)t(1;5)(p34;q15), der(11)ins(11;5)(p13;q15q32)	*CAPRIN1*(*GPIAP1*)-*PDGRFB* (*CAPRIN1* is at 11p13)	CEL
t(5;12)(q32;q24.1)	*GIT2-PDGFRB*	CEL
Uninformative cytogenetics, t(5;12)(q32;q23.3) predicted	*SART3-PDGFRB* (and *PDGFRB-SART3*)	MPN with eosinophilia and myelofibrosis [174]
t(5;12)(q32;p13.3)	*ERC1-PDGFRB*	AML (without eosinophilia) [175]
t(5;12)(q32;q13.1)	*BIN2-PDGFRB*	aCML with eosinophilia [162]
t(5;12)(q32;q15)	*CPSF6-PDGFRB*	CMML with eosinophilia [163]
t(5;14)(q32;q22.1)	*NIN-PDGFRB*	aCML (13% eosinophils)

(*Continued*)

Table 6.5 (Continued)

Chromosomal rearrangement	Fusion gene	Haematological presentation
t(5;14)(q32;q32.1)	*TRIP11*(*CEV14*)-*PDGFRB*	Occurred at relapse of AML, associated with appearance of eosinophilia [176]
t(5;14)(q32;q32.1) or variant t(5;17;14)(q32;q11;q32.1)	*CCDC88C*(*KIAA1509*)-*PDGFRB*	CMML with eosinophilia [177]
t(5;15)(q32;q15.3)	*TP53BP1-PDGFRB*	aCML with prominent eosinophilia
t(5;16)(q32;p13.1)	*NDE1-PDGFRB*	CMML with eosinophilia
t(5;17)(q32;q11.2)	*MPRIP-PDGFRB*	CEL [163]
t(5;17)(q32;p11.2)	*SPECC1-PDGFRB*	JMML with eosinophilia
t(5;17)(q32;p13.2)	*RABEP1-PDGFRB*	CMML (without eosinophilia); MDS/MPN, unclassified plus T-LBL, with evolving eosinophilia [171]; T-LBL with MPN but no eosinophilia [108]
t(5;17)(q32;q11.2)	*MYO18A-PDGFRB*	MPN with eosinophilia [178]
t(5;17)(q32;q21.3) or t(5;17;22)(q32;q21.3;q21)	*COL1A1-PDGFRA*	MDS with eosinophilia, MPN with eosinophilia [179]
t(5;19)(q32;p13)	Partner gene not identified	aCML with eosinophilia [180]
t(5;20)(q32;p11.2)	*DTD1-PDGFRB*	CEL [177]

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, myelodysplastic syndrome; MDS/MPN, myelodysplastic/myeloproliferative neoplasm; MPN, myeloproliferative neoplasm; T-LBL, T lymphoblastic lymphoma.

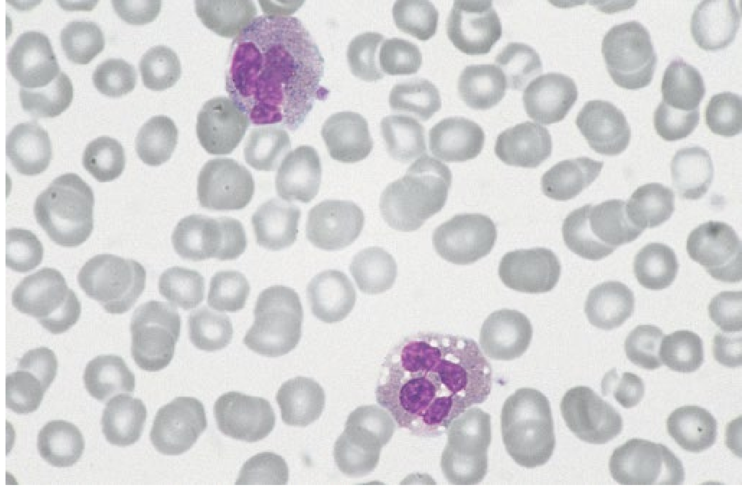


Fig. 6.18 PB film from a patient with chronic eosinophilic leukaemia with t(5;12)(q32;p13.2). One of the eosinophils is vacuolated but overall cytological abnormalities were fairly minor. (With thanks to Dr Elisa Granjo, Porto.)

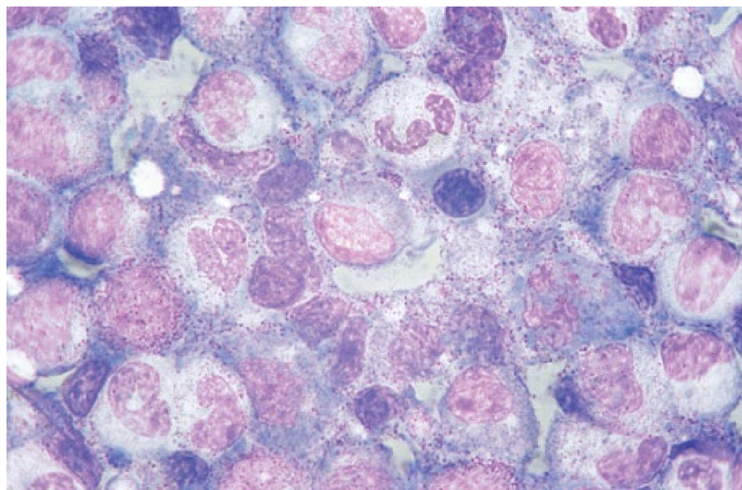


Fig. 6.19 BM film from a patient with chronic eosinophilic leukaemia with t(5;12)(q32;p13.2) showing a marked increase in eosinophils and precursors (same patient as Fig. 6.18).

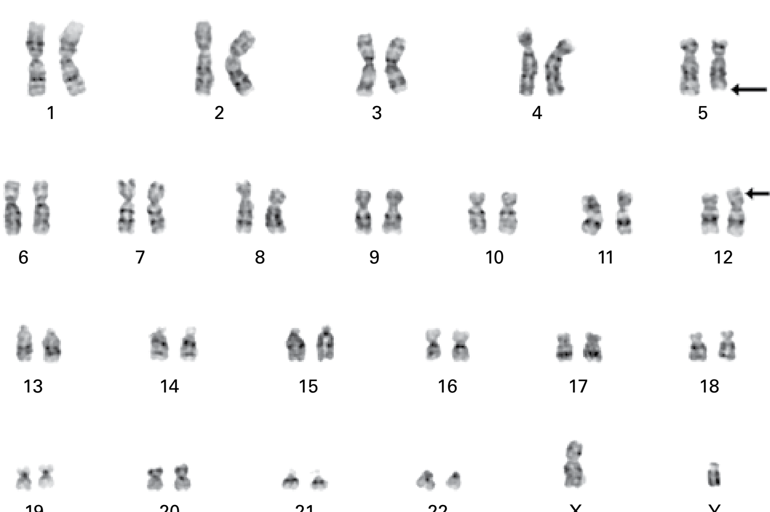


Fig. 6.20 Karyogram of a patient with chronic eosinophilic leukaemia with t(5;12)(q32;p13.2) (same patient as Figs 6.18 and 6.19). (With thanks to Sofia Dória, Porto.)

haematological neoplasm but with specified rearrangements associated with B-ALL being excluded. The most frequent cytogenetic abnormality is t(5;12)(q32;p13.2) (Fig. 6.20). The 5q breakpoint appears variable because these rearrangements are often complex and FISH analysis may be misleading [157]. Other reported chromosomal abnormalities are summarized in Table 6.5. If cytogenetic analysis fails, break-apart FISH can be employed. The eosinophils are part of the leukaemic clone [186]. As in the case of CML, development of a clonal cytogenetic abnormality (trisomy 8) during imatinib therapy in cells that were not part of the leukaemic clone has been reported [187]. Occasionally cytogenetic analysis is normal but *PDGFRB* rearrangement is found on FISH analysis [168,179].

Problems and pitfalls

Because very effective treatment is available it is important to recognize this syndrome. The reported 5q breakpoints have varied. 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 8p11.2, previously referred to as the 8p11 syndrome, form a heterogeneous group of lymphoid and myeloid neoplasms with prominent eosinophilia (Table 6.6) [2,103,188–206]. There is a slight male preponderance and a relatively young age of presentation (median age of reported patients 32 years) [190]. Presenting disease features include splenomegaly and lymphadenopathy, the latter due either to infiltration by myeloid cells or to T lymphoblastic lymphoma. The prognosis is poor although some patients have survived following bone marrow transplantation. A single patient with a *BCR-FGFR1* fusion had a partial remission with ponatinib [206], but in general early transplantation is considered indicated in patients with *FGFR1* rearrangement.

Clinical and haematological features

Clinical presentation is very variable, often with fever, weight loss and night sweats, sometimes with lymphadenopathy or a mediastinal mass and sometimes with splenomegaly. The haematological features may be those of an MPN with eosinophilia (Figs 6.21 and 6.22), with or without simultaneous T lymphoblastic leukaemia/lymphoma (Fig. 6.23) or, rarely, B lymphoblastic leukaemia/lymphoma [207]. Presentation may be as AML, with eosinophilia reported in about half of these patients [208]. Mixed phenotype acute monoblastic/precursor B lymphoblastic leukaemia with eosinophilia has been described [209]. A mixed phenotype T-B-myeloid neoplasm with eosinophilia and a T lymphoblastic lymphoma has also been described [210]. Eosinophilia is not necessarily part of the syndrome. Sometimes basophils, neutrophils or monocytes are increased. 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 [207,211–213]. T lymphoblasts, B lymphoblasts and myeloid cells belong to the cytogenetically abnormal neoplastic clone. At least five patients have been reported with polycythaemia as part of the syndrome, all associated with t(6;8)(q27;p11.2) and in one case with a coexisting *JAK2* V617F mutation [214,215]. Basophilia may be a feature when there is *BCR-FGFR1* or *TPR-FGFR1*. Mast cells may be increased, spindle-shaped [108,216] and CD25-positive. In one patient they were demonstrated to be part of the neoplastic clone [217]. Another patient has been reported with urticaria pigmentosa progressing to systemic mastocytosis followed by transformation to Ph-negative aCML with t(8;17)(p11;q25); however, no molecular analysis to confirm *FGFR1* involvement was possible in this case [218]. In another patient with t(8;13) and *ZMYM2-FGFR1* the bone marrow was infiltrated by dysplastic CD25-positive mast cells and the WHO criteria for systemic mastocytosis were met; however, the D816V *KIT* mutation was not detected [199]. In a third patient with blastic transformation of atypical chronic myeloid leukaemia (without eosinophilia) with t(8;19)(p11.2;q13.3) there was coexisting *KIT*-mutated systemic mastocytosis [201].

Cytogenetic and molecular genetic features

The majority of cases have been associated with t(8;13)(p11.2;q12.1) (Figs 6.24 and 6.25), t(8;9)(p11.2;q33.2) or t(6;8)(q27;p11.2). The most frequent cytogenetic association is with t(8;13)(p11.2;q12.1) and *ZMYM2-FGFR1* (previously *ZNF98-FGFR1*), observed in about half of cases; an associated submicroscopic deletion of *FGFR1* leading to absence of *FGFR1-ZMYM2* is observed [219]. Other translocations and fusion genes that may underlie this syndrome are shown in Table 6.6.

Table 6.6 Lymphoid and myeloid neoplasms associated with rearrangement of *FGFR1* [2,102,186–203]; the most common rearrangement is shown in bold*.

Cytogenetics	Molecular genetics	Number†	Haematological syndromes
t(1;8)(q31.1;p11.2)	*TPR-FGFR1*	1	aCML with neutrophilia, eosinophilia and basophilia plus T lymphoblastic lymphoma [191]; CMML with eosinophilia and T lymphoblastic lymphoma [192]
t(2;8)(q13;p11.2)	*RANBP2-FGFR1*	1	aCML with eosinophilia [193]
t(2;8)(q37.3;p11.2)	*LRRFIP1-FGFR1*	1	Appeared at transformation of RAEB to AML with eosinophilia [194]
t(6;8)(q27;p11.2)	*FGFR1OP-FGFR1*	At least 7	CEL, MPN-U, T-ALL, AML, B-lineage ALL, polycythaemia in five patients
t(7;8)(q22.1;p11.2)	*CUX1(CUTL1)-FGFR*	1	T lymphoblastic leukaemia/lymphoma without eosinophilia [195]
t(7;8)(q33;p11.2)	*TRIM24-FGFR1*	1	AML with eosinophilia, AML without eosinophilia relapsing as MPN with eosinophilia [196]
inv(8)(p11.2q13)	Partner gene not identified	1	AML without eosinophilia [197]
t(8;9)(p11.2;q33.2)	*CNTRL(CEP110)-FGFR1*	At least 9	CEL, CMML, AMML following primary myelofibrosis, T-ALL/T-LBL; sometimes there is monocytosis or thrombocytosis
t(8;11)(p11;p15)	*NUP98-FGFR1* (postulated)	1	
t(8;12)(p11.2;q15)	*CPSF6-FGFR1*	1	T non-Hodgkin lymphoma with mild eosinophilia (eosinophils 0.8×10^9/l) [198]
t(8;13)(p11.2;q12.1)	***ZMYM2(ZNF198)-FGFR1***	**More than 21**	**CEL, T-ALL/T-LBL, AML, B-lineage ALL, AML plus T-LL; blastic transformation of aCML with eosinophilia with 'systemic mastocytosis' (*KIT* mutation not detected)** [199]
t(8;17)(p11.2;q11.2)	*MYO18A-FGFR1*	1	aCML with severe thrombocytopenia
t(8;19)(p11.2;q13.3)	*HERVK-FGFR1*	3	AML with 45% bone marrow eosinophils; AMML following CMML without eosinophilia [200]; aCML (with *KIT*-mutated systemic mastocytosis) [201]
t(8;22)(p11.2;q11.2)	*BCR-FGFR1*	12+	Can resemble Ph-positive CML; myeloid transformation may occur; one T + B lymphoid transformation; one transformation to B-ALL [202]; AML [203]; B-ALL; one B/myeloid acute leukaemia [204]; one trilineage acute leukaemia/T lymphoblastic lymphoma [205]; one B/T/myeloid acute leukaemia [206]; aCML without eosinophilia [200]; eosinophilia is not usually a feature
ins(12;8)(p11.2;p11p22)	*FGFR1OP2-FGFR1*	1	T-LBL and mild eosinophilia that progressed rapidly to AML

aCML, atypical chronic myeloid leukaemia; ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; AMML, acute myelomonocytic leukaemia; CEL, chronic eosinophilic leukaemia; CMML, chronic myelomonocytic leukaemia; MPN, myeloproliferative neoplasm; RAEB, refractory anaemia with excess of blasts; T-ALL, T-lineage acute lymphoblastic leukaemia; T-LBL, T-lineage lymphoblastic lymphoma; T-LL, T-lineage lymphoblastic lymphoma.

* In addition, *FGFR1* rearrangement has been found in association with t(8;12)(p11.2;q15) associated with T lymphoblastic lymphoma and MPN, and with t(8;17)(p11.2;q25) associated with CML and systemic mastocytosis but the suspected involvement of *FGFR1* in t(8;11)(p11;p15) was not confirmed.

† Modified from reference 2. Numbers updated to 2008 from reference 190.

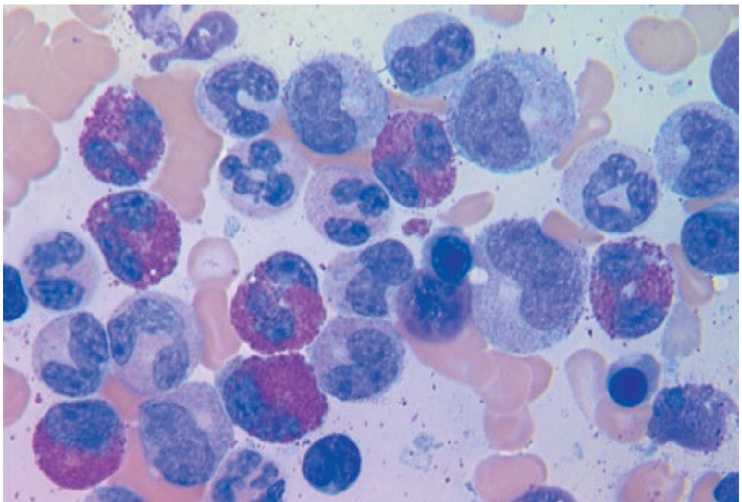


Fig. 6.21 BM film from a 27-year-old man with a myeloid and lymphoid neoplasm (chronic eosinophilic leukaemia and T lymphoblastic lymphoma) associated with t(8;13)(p11.2;q12.1) showing an increase of neutrophils, neutrophil precursors, eosinophils and eosinophil precursors. H&E ×100. (With thanks to Dr Colm Keane, Princess Alexandra Hospital, Brisbane.)

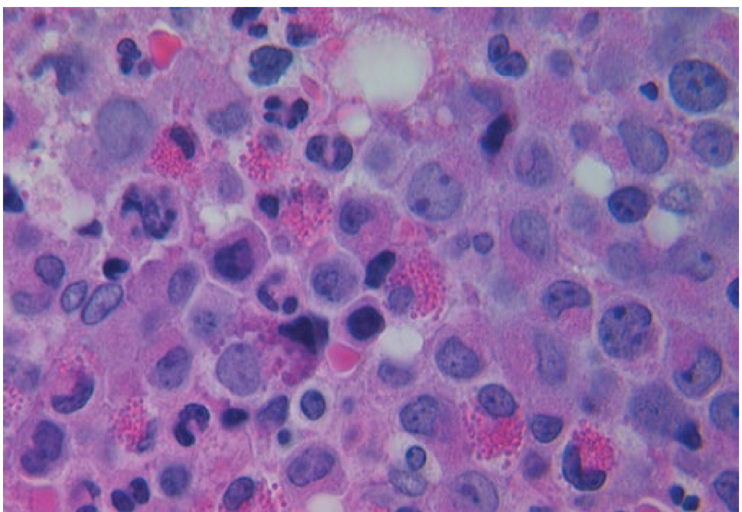


Fig. 6.22 BM trephine biopsy section from a patient with a myeloid and lymphoid neoplasm associated with t(8;13)(p11.2;q12.1) (same patient as Fig. 6.21) showing hypercellularity and an increase in eosinophils and precursors. H&E ×100. (With thanks to Dr Colm Keane.)

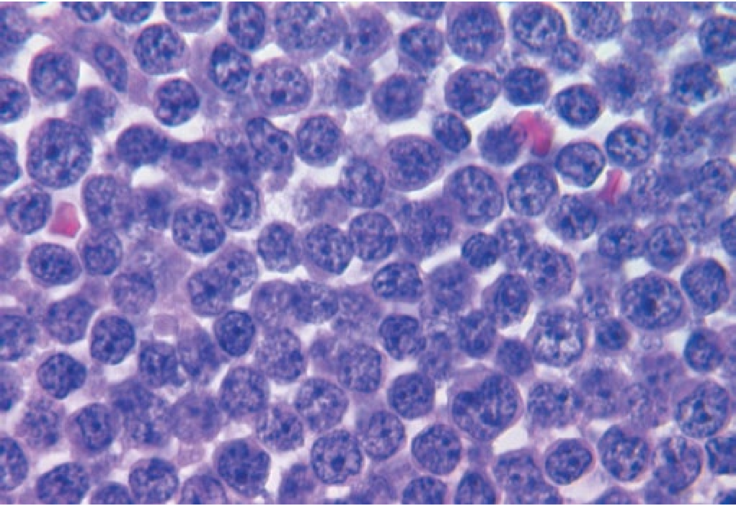


Fig. 6.23 Lymph node biopsy from a patient with a myeloid and lymphoid neoplasm associated with t(8;13)(p11.2;q12.1) showing lymphoblastic infiltration of a lymph node (same patient as Figs 6.21 and 6.22). H&E ×100. (With thanks to Dr Colm Keane.)

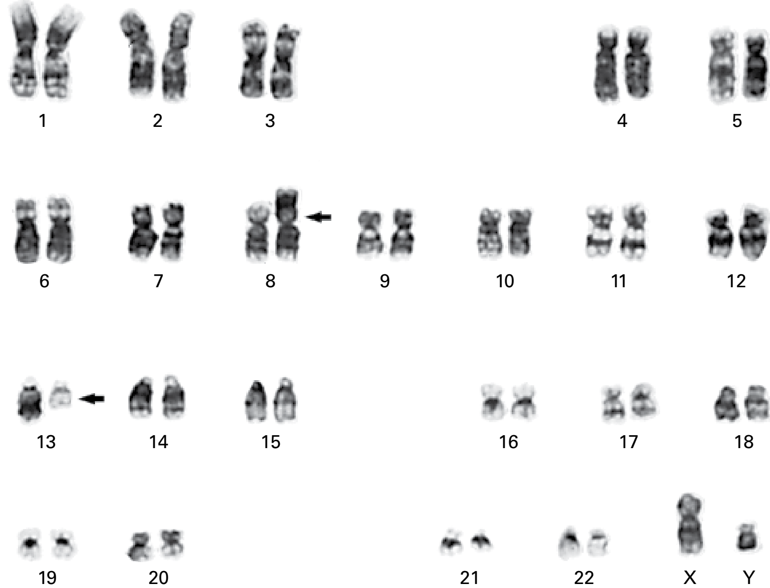


Fig. 6.24 Karyogram showing t(8;13)(p11.2;q12.1). (With thanks to Jill Elliott and the Regional Cytogenetics Service, Sheffield Children's Hospital.)

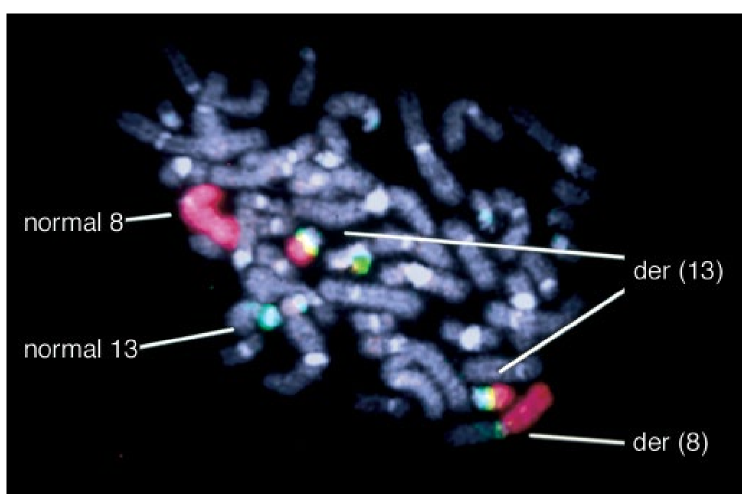


Fig. 6.25 FISH analysis from a patient with an MPN associated with t(8;13)(p11.2;q12.1); in this patient there was subsequently transformation to acute myeloid leukaemia. (With thanks to Dr Donald Macdonald and Dr A. Chase, Hammersmith Hospital.)

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.

Myeloid and lymphoid neoplasms associated with *PCM1-JAK2*

Haematological neoplasms associated with t(8;9)(p22;p24.1) and *PCM1-JAK2* are recognized as a provisional entity in the 2016 revision of the WHO classification [2,220,221]. The features may resemble those of a MPN, MDS/MPN or AML (Fig. 6.26) [221]. Presentation with B-ALL and T lymphoblastic lymphoma have been reported, as has B lymphoblastic transformation – following acquisition of t(8;22) – indicating an origin from a haemopoietic stem cell.

A small number of cases have been reported with t(9;12)(p24.1;p13.2) and *ETV6-JAK2* and a myeloid, B-cell precursor or T-cell precursor neoplasm. Similarly a small number of cases have been reported with a myeloid or B-cell precursor neoplasm associated with t(9;22)(p24.1;q11.2) and *BCR-JAK2*. Although less well

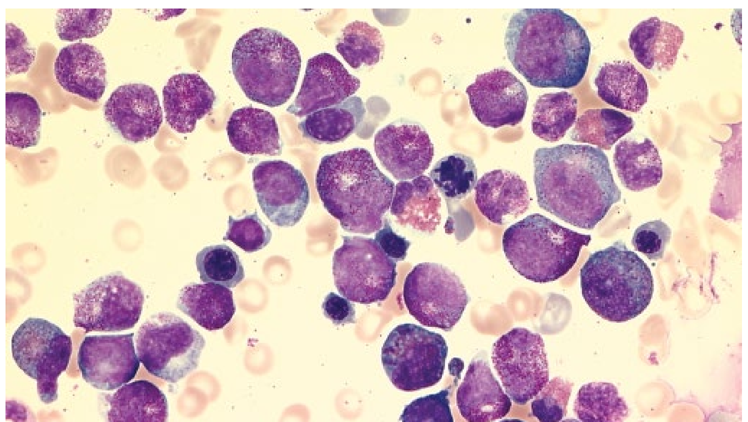


Fig. 6.26 BM film from a patient with *PCM1-JAK2* showing increased eosinophils and precursors. MGG ×100. (With thanks to Dr Torsten Haferlach, Munich.)

defined, both these groups of disorders may be considered variants of this entity. However, the cases of B-ALL can have features of Ph-like ALL and may be better categorized as such.

Detection of t(8;9) translocation is important since a complete cytogenetic response has been reported with ruxolitinib, a JAK2 inhibitor [222]; sometimes sustained complete remission is achieved but other patients have only a partial remission or remission is not sustained [223].

Clinical and haematological features

There is a marked male predominance (M : F = 5 : 1) and a wide age range, from childhood to old age. Clinical features often include hepatosplenomegaly. Haematological features are very variable. Patients presenting as an MPN usually have the features of CEL but sometimes of primary myelofibrosis. Those with MDS/MPN often have the features of aCML, sometimes with prominent eosinophilia. Monocytosis and basophilia can occur but are not common. In those presenting in chronic phase, myeloblastic transformation or B lymphoblastic transformation can occur. A bone marrow aspirate often shows dysplasia, particularly erythroid dysplasia, and on trephine biopsy there may be fibrosis and sheets of immature erythroid cells, simulating erythroleukaemia [2].

The haematological features associated with *ETV6-JAK2* are heterogeneous: B-ALL, T-ALL, aCML or MDS. The features associated with *BCR-JAK2* are similarly heterogeneous, mainly B-ALL or aCML but sometimes AML.

Cytogenetic and molecular genetic features

The majority of the more than 30 cases reported have had t(8;9)(p22;p24.1) and *PCM1-JAK2.* Cases with *ETV6-JAK2* have t(9;12)(p24.1;p13.2) or a variant translocation, often with additional cytogenetic abnormalities. *BCR-JAK2* similarly can result from t(9;22)(p24.1;q11.2) or a variant translocation or insertion; sometimes there are additional abnormalities.

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 [224] are classified as chronic basophilic leukaemia. Patients whose neoplastic cells have *PDGFRB* rearrangement are also excluded from this category. Chronic basophilic leukaemia is very rare. In one small series two of four patients had possible concurrent mast cell disease [225]. Acute transformation can occur [225].

Clinical and haematological features

Clinical features are splenomegaly and sometimes signs and symptoms consequent on histamine excess [225,226]. The peripheral blood

and bone marrow show dominant basophilic differentiation. There may also be eosinophilia [225]. Basophils can be specifically identified by flow cytometry or immunohistochemistry using the 2D7 monoclonal antibody, which recognizes a granule protein.

Cytogenetic and molecular genetic features

By definition, cases are Ph negative and do not have *BCR-ABL1* fusion. Cytogenetic abnormalities observed have included monosomy 7 and trisomy 8 with del(11q) [225].

Conclusions

The chronic myeloid leukaemias include conditions that are primarily proliferative (MPN in the WHO classification) and other conditions that also have dysplastic features and thus are classified as MDS/MPN in the WHO classification (see Chapter 5). 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 effective 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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7

Lymphoid Leukaemias of Mature B, T and Natural Killer Cells

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Diagnosis and classification of lymphoid leukaemias and leukaemic-phase lymphomas of mature T, B and natural killer (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 that, although 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 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 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 2016 revision of 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 has only a minor role.

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, chronic lymphocytic leukaemia (CLL) and a number of other 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, that is 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 (Tables 7.1 and 7.2) [4–6]. 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: (i) assessment of lineage; (ii) presumptive demonstration of clonality; (iii) recognition of immunophenotypic characteristics that distinguish the chronic lymphoproliferative disorders from ALL; (iv) demonstration of patterns of antigen expression that support a specific diagnosis; (v) recognition of different patterns of antigen expression within a specific disease category that are of prognostic significance, for example 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 CLL; (vi) confirmation of expression of a specific antigen (e.g. CD19, CD20 or CD52) when a McAb is to be used in therapy; and (vii) monitoring of MRD, for example in CLL monitoring of CD19+, CD5+, 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 (TP53) 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 7.1 Panel of monoclonal antibodies recommended by the British Committee for Standards in Haematology 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.

Table 7.2 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, CD138

NK, natural killer; TCR, T-cell receptor.

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 underexpress 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, for example 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. 7.1 [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 difficult.

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.

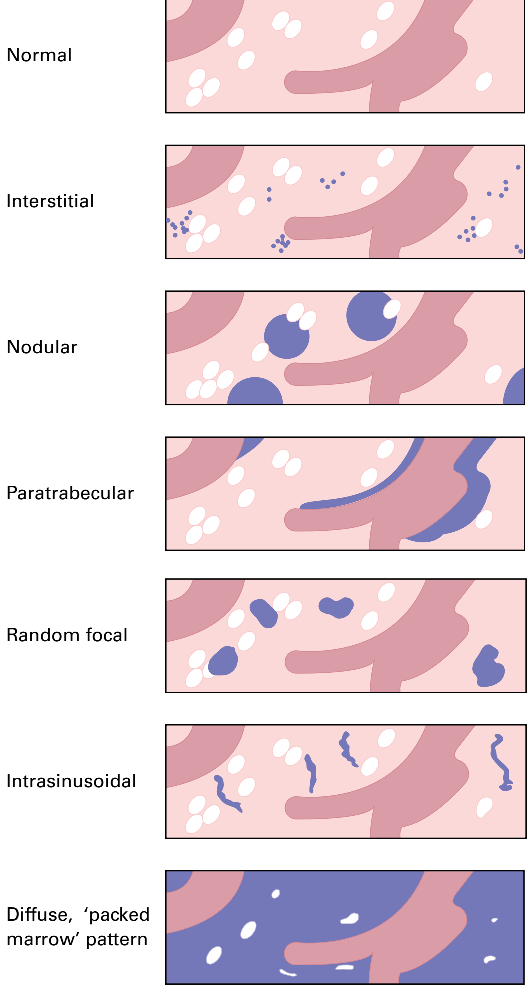


Fig. 7.1 Patterns of bone marrow (BM) infiltration observed in lymphoproliferative disorders. (Reproduced with permission from Bain *et al.* 2010 [8].)

Molecular genetic analysis is useful in establishing clonality by the detection of immunoglobulin (IGH) or T-cell receptor (TCR) loci 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 (mRNA) transcripts. Immunocytochemistry and immunohistochemistry can be regarded as extensions of molecular genetic techniques when they 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 7.3.

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 can be staged by the Ann Arbor staging system, initially proposed for Hodgkin lymphoma (Table 7.4; Fig. 7.2), or by a modification of it [11]. By definition, lymphomas that involve the peripheral blood are stage IV. A different staging system is

Table 7.3 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* (13q14.2), *DBM* (13q14) or *BRCA2* (13q13.1)	50%
del(11)(q22.3)	Deletion of *ATM* at 11q22.3	20%
Trisomy 12	Unknown	20%
del(17)(p13.1)	Deletion of *TP53* at 17p13.1	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;q21.3)	Dysregulation of *BCL2* at 18q21.3 by proximity to IGH locus	70–90%
t(2;18)(p11.2;q21.3)	Dysregulation of *BCL2* at 18q21.3 by proximity to κ locus	
t(18;22)(q21.3;q11.2)	Dysregulation of *BCL2* at 18q21.3 by proximity to λ locus	

(Continued)

Table 7.3 (Continued)

Cytogenetic abnormality	Associated molecular genetic abnormality	Approximate frequency (where known)
Mantle cell lymphoma		
t(11;14)(q13.3;q32)	Dysregulation of *CCND1* at 11q13.3 by proximity to IGH locus, with consequent overexpression of cyclin D1 in the nucleus	90%
Lymphoplasmacytoid lymphoma		
None	Mutation of *MYD88* at 3p22.2	Majority
t(9;14)(p13.2;q32)	Dysregulation of *PAX5* at 9p13.2 by proximity to IGH locus	Minority and not specific
Burkitt lymphoma		
t(8;14)(q24.2;q32)	Dysregulation of *MYC* at 8q24.2 by proximity to IGH locus	70–80%
t(2;8)(p11.2;q24.2)	Dysregulation of *MYC* at 8q24.2 by proximity to κ locus	
t(8;22)(q24.2;q11.2)	Dysregulation of *MYC* at 8q24.2 by proximity to λ locus	
T-cell prolymphocytic leukaemia		
inv(14)(q11.2q32.1), t(14;14)(q11;q32)	*TCL1A* and *TCL1B* at 14q32.1 dysregulated by proximity to TRA/TRD locus	75%
t(X;14)(q28;q11.2)	*MTCP1* at Xq28 dysregulated by proximity to TRA/TRD locus	
t(X;7)(q28;q34)	*MTCP1* at Xq28 dysregulated by proximity to TRB locus	

Table 7.4 Staging of Hodgkin and non-Hodgkin lymphoma (Lugano modification of Ann Arbor staging)*.

Stage	Criteria
I	One node or a group of adjacent nodes OR single extranodal lesion without nodal involvement (IE)
II	Two or more nodal groups on the same side of the diaphragm (there may be limited contiguous involvement) or as for stage I but with limited contiguous involvement
III	Nodes on both sides of the diaphragm; nodes above the diaphragm with spleen involvement
IV	Additional non-contiguous extralymphatic involvement, e.g. spread 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 >38.3 °C

* Involvement is assessed by physical examination, computed tomography (CT) scanning, often ^{18}F-fluorodeoxyglucose positron emission tomography (FDG-PET) and sometimes magnetic resonance imaging (MRI).

† Patients with Hodgkin lymphoma are given a composite stage, e.g. IA, IIIB, but B symptoms are no longer considered relevant to staging and prognostication in non-Hodgkin lymphoma [11].

Adapted from reference 11.

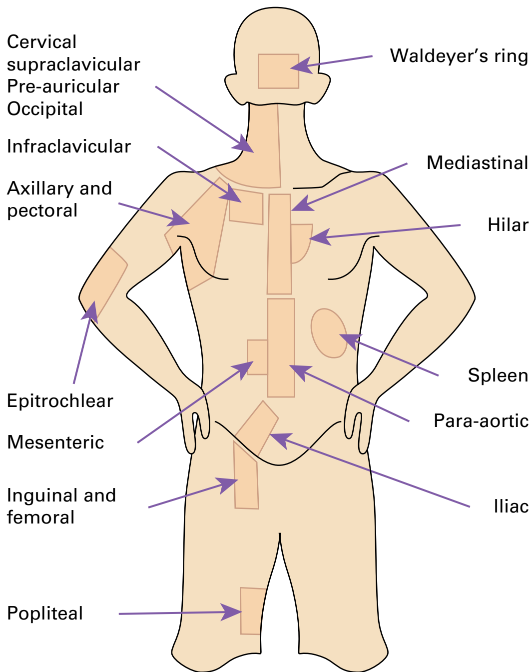


Fig. 7.2 Lymph node regions used for staging of Hodgkin and non-Hodgkin lymphoma.

Table 7.5 International Prognostic Index for high-grade non-Hodgkin lymphoma.

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.
Derived from reference 12.

applied to CLL (see below) and also to Burkitt lymphoma and to cutaneous lymphomas.

Various prognostic scoring systems have been proposed, for example for aggressive non-Hodgkin lymphoma [12] (Table 7.5) and for follicular lymphoma [13] (Table 7.6).

Table 7.6 Follicular Lymphoma International Prognostic Index (FLIPI).

Criterion	Score
Age >60 years	1
Ann Arbour 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.
Derived from reference 13.

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 7.7). 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 lymphocytes 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 [14], whereas lymphoblasts do not.

DNA analysis shows that, in B-lineage chronic lymphoid leukaemias, the heavy chain

Table 7.7 Some monoclonal antibodies used in the characterization of chronic lymphoid leukaemias of B lineage.

Cluster	Specificity within haemopoietic and lymphoid lineages or other specificity
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 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 lymphomas, 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 neoplasms; expressed on a small subset of normal B cells, in a majority of cases of 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	Intraepithelial lymphocytes, small subset of peripheral blood lymphocytes, hairy cells
CD123	Hairy cells, blastic plasmacytoid dendritic cell neoplasm
CD138	Plasma cells including myeloma cells
CD200	CLL, HCL, B-ALL, most multiple myeloma but not follicular lymphoma, mantle cell lymphoma or Burkitt lymphoma and not usually B-PLL
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 [14])
HLA-DR*	Virtually all B lymphocytes and their precursors, activated T cells, haemopoietic precursors, monocytes
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-NHL, B non-Hodgkin lymphoma; B-PLL, B-prolymphocytic leukaemia; CLL, chronic lymphocytic leukaemia; 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.

and usually the light chain immunoglobulin loci have undergone rearrangement; in some cases the T-cell receptor β (TRB) 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 7.8.

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 antigen-experienced, activated B cell, which in some patients is a post-germinal centre B cell that has undergone somatic hypermutation of *IGHV* (IGH variable region) 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 [15]. Some patients with small lymphocytic lymphoma show disease evolution to CLL. In most, if not all, patients CLL is preceded by monoclonal B-cell lymphocytosis (see page 445). In a retrospective study of 45 patients for whom

Table 7.8 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, CD20, CD24, CD79a	++*	++	++†	++	++	++	–
CD79b	–	++	–/+	++	++	++	–
CD23	++	–	–	–/+	–/+	–/+	–
FMC7, CD22	–/+	++	++	++	+	++	–
CD10	–	–/+	–	+	–/+	–	–/+
CD11c	–/+	++	++	–	–/+	+	?
CD25	–/+	–	++	–	–	–/+	–
CD38	–/+	–	–/+	–/+	–	–/+	++
CD200	++	–	++	–	–	–	–/+
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.

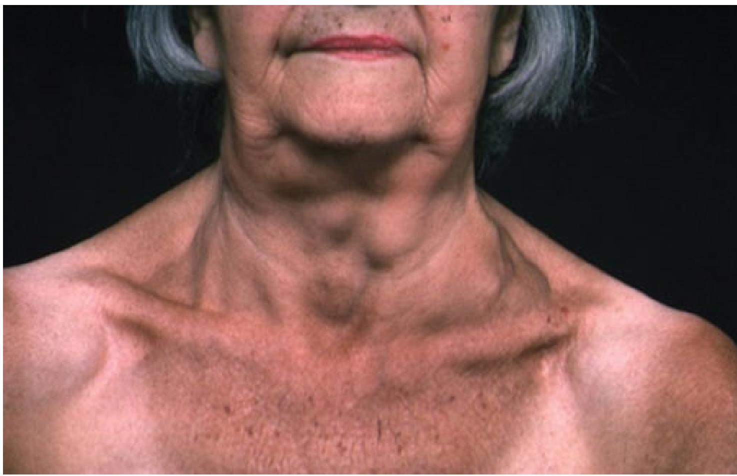


Fig. 7.3 Clinical photograph of a patient with chronic lymphocytic leukaemia (CLL) showing cervical lymphadenopathy.

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 [16]. However, prolonged lymphocytopenia, possibly representing reduced numbers of T lymphocytes, has also been reported preceding CLL [17]. Other immunological abnormalities that may precede CLL by up to 9 years include an abnormal free light chain ratio (in 38% of 109 patients), a paraprotein (in 13%) and hypogammaglobulinaemia (in 3%) [18]. A minority of patients show polyclonal B-cell activation [18].

Mu 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, North American Indians and African Americans and is higher in Jews. The incidence in Taiwan has increased markedly over the last 20 years suggesting a possible effect of a more Western lifestyle [19]. The incidence in those of African ancestry is intermediate between the rates in Asian and White populations [20]. The prognosis is worse in South Asians (Indians) and onset may be at a younger age [21]. 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. The disease runs a more benign course and is more responsive to treatment in women [22]. Adolescent cases are very rare [23]. Some cases are familial, the pattern of inheritance suggesting a dominantly acting gene [24]. In some families there is linkage to 13q21.33-q22.2 [25]. A meta-analysis identified polymorphisms on four genes as being likely to be of relevance: *ABCB1* (*MDR1*), *LTA*, *CD38* and *IFNGR1* [26]. In the later stages, CLL is characterized by lymphadenopathy (Fig. 7.3), 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 (e.g. $>10 \times 10^9$/l) but the demonstration of a

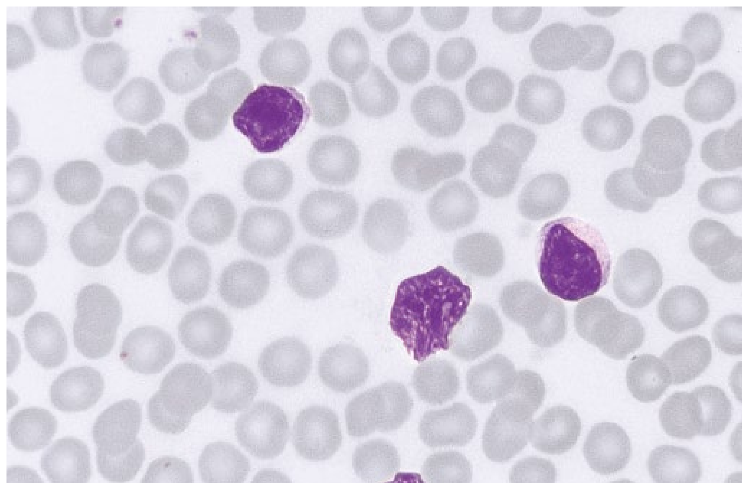
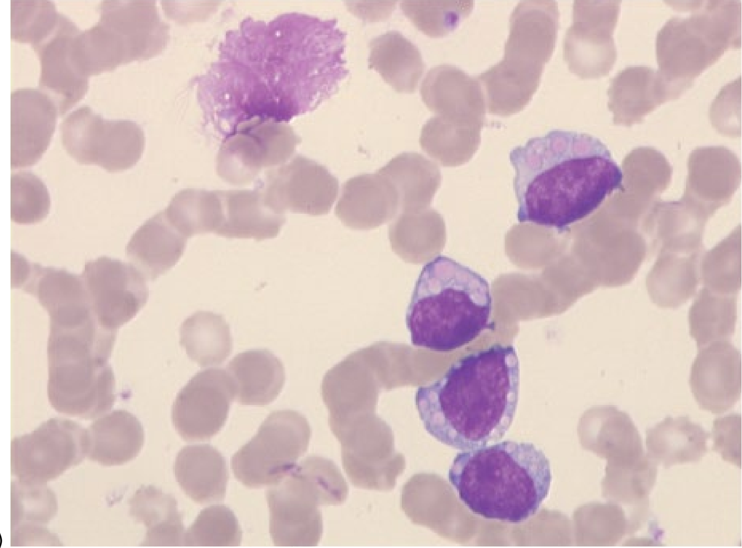


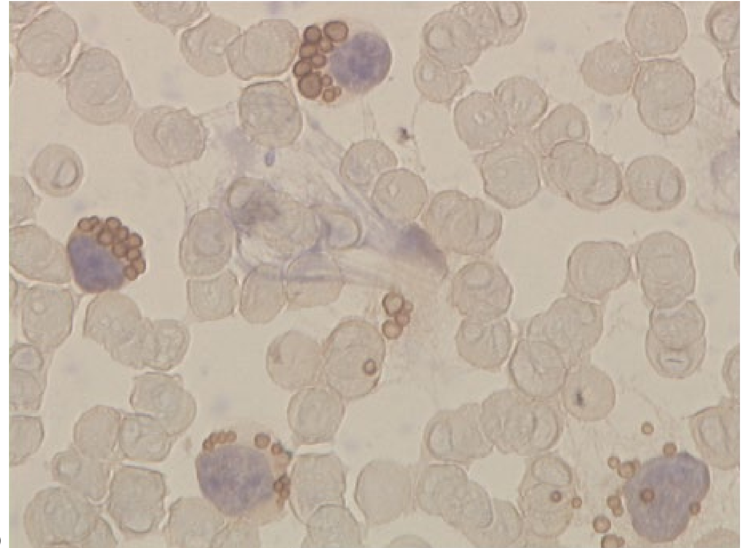
Fig. 7.4 Peripheral blood (PB) film in CLL showing two mature lymphocytes and one smear cell. Nuclear chromatin is condensed and each cell contains a barely detectable nucleolus. May–Grünwald–Giemsa (MGG) ×100.

monoclonal population of B lymphocytes with a characteristic immunophenotype permits diagnosis at an earlier stage when the lymphocyte count is less elevated. The 2016 revision of the WHO classification requires a minimum of at least 5×10^9/l clonal B cells with characteristic cytological and immunophenotypic features, with the lymphocytosis persisting for at least 3 months [15]. It has been suggested that a diagnosis of CLL rather than small lymphocytic lymphoma should be made when the lymphocyte count is lower but there is cytopenia due to bone marrow infiltration [27]. Others have suggested that the diagnostic threshold should be a B lymphocyte count of 11×10^9/l with a CLL immunophenotype, since this count better separates patients who are likely to survive without needing treatment from those who are not [28], but this has not found general acceptance. Some patients have a paraprotein, usually immunoglobulin (Ig) M, present in low concentration, and as disease advances there is hypogammaglobulinaemia. Spontaneous remission can occur in CLL but this is rare [29]; when it occurs, the leukaemic clone may reduce to minimal levels [29].

The leukaemic cells in CLL are typically small with a conspicuous though usually narrow rim of cytoplasm (Fig. 7.4). 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, granules, vermiform inclusions, globular inclusions (Fig. 7.5) or crystals (seen as negative images) (Fig 7.6), which represent immunoglobulin [30–32]. 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 reduced CD45 expression [33], hypermutated *IGHV* genes [34] (see below) and a better prognosis [33,34]. The presence of up to 55% of prolymphocytes (see below for description) is compatible with the diagnosis of CLL but only 2% of patients have 15% or more [35]. 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



(a)



(b)

Fig. 7.5 PB film from a patient with CLL showing: (a) a smear cell and four lymphocytes containing globular immunoglobulin inclusions; (b) immunocytochemistry showing expression of immunoglobulin M. (With thanks to Professor Georgia Metzgeroth and Dr Jan Haskta, Mannheim.)

of lymphoplasmacytoid lymphocytes or cells with cleft nuclei; their presence correlates with a worse prognosis [29]. In a rare morphological variant, binucleated lymphocytes comprise a significant minority of cells [36]. Other rare features are nuclear budding, micronuclei and internuclear bridges [37].

In the early stages of the disease the peripheral blood abnormality is confined to the lymphocytes. An incidental eosinophilia in a blood count is occasionally found to be associated with previously unrecognized CLL [38]. 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 than 1 year) are of

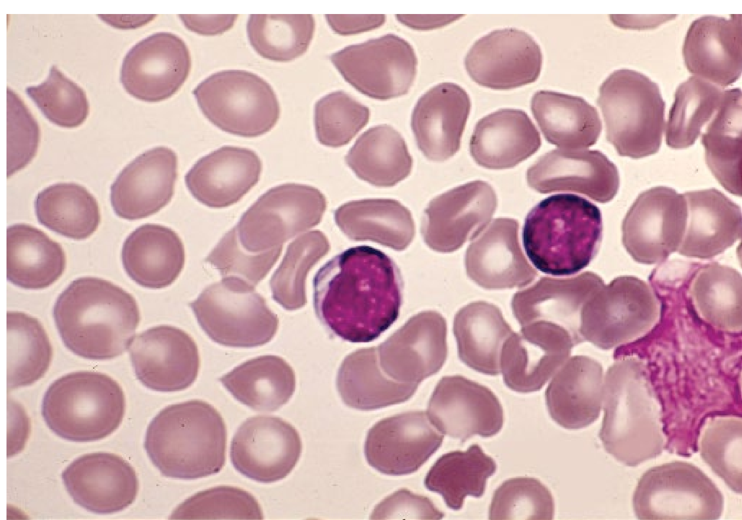


Fig. 7.6 PB film from a patient with CLL showing the negative image of an immunoglobulin crystal.

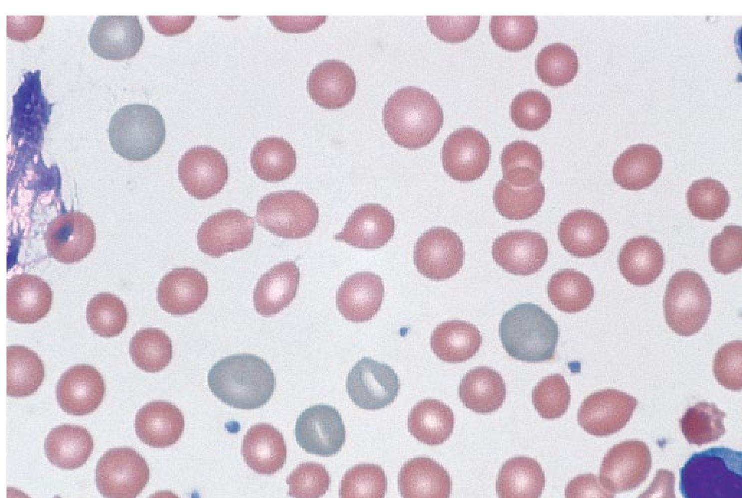


Fig. 7.7 PB film in CLL complicated by autoimmune haemolytic anaemia showing one mature lymphocyte, one smear cell, spherocytes and polychromatic macrocytes. MGG ×100.

prognostic significance [39]. 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 [40]. Ibrutinib therapy is associated with a striking increase in the lymphocyte count, on average three-fold and peaking at 4 weeks, as lymphocytes are mobilized from tissues [41]. 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. 7.7). Either the development of AIHA or the presence of a positive direct antiglobulin test was independently predictive of a worse survival in one study [42], but another very large study found that autoimmune complications did not worsen survival [43]. Autoimmune thrombocytopenia is associated more with earlier stages of the

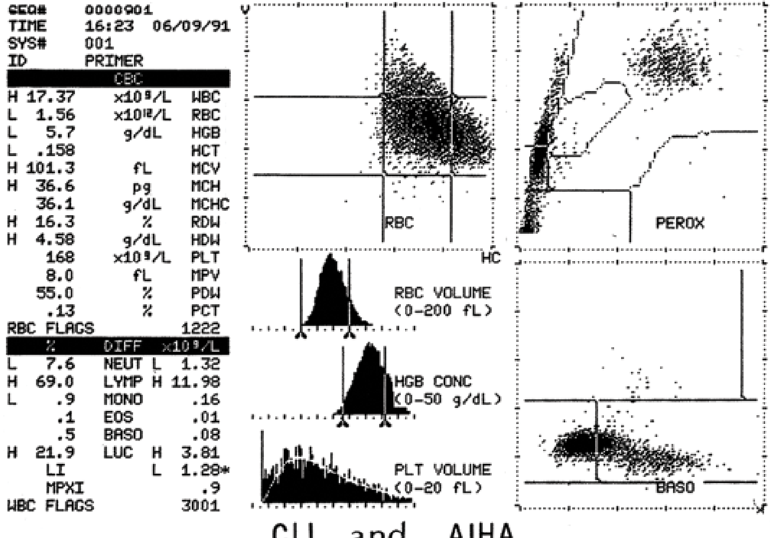


Fig. 7.8 Histograms and scatterplots on a Bayer-Technicon H2 automated counter of peripheral blood from a patient with CLL complicated by autoimmune haemolytic anaemia. In the peroxidase channel scatterplot the neoplastic lymphocytes form a fusiform cluster extending from the 'debris' area (bottom left) 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.

disease, occurring at a median of 13 months from diagnosis in one study [44], and may be the presenting feature [45]. The blood count and film show reduction of platelet numbers out of proportion to the degree of anaemia. The development of autoimmune thrombocytopenic purpura was an independent adverse prognostic factor in one study [44]. Occasional patients develop Evans syndrome, that is 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 [42]. Pure red cell aplasia is a less recognized association of CLL, which may be under-reported [45]; it may be autoimmune in origin or the result of a chronic parvovirus B19 infection [46]. 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 [47]. Pure white cell aplasia is a rare occurrence [48]; one case has been reported in association with fludarabine therapy [49]. Automated full blood counters produce characteristic scatterplots and histograms in patients with CLL (Figs 7.8 and 7.9).

Bone marrow aspiration and trephine biopsy (see below) are not necessary for diagnosis of CLL but are recommended in the investigation of cytopenia and before initiation of therapy. They should also be performed if the immunophenotype is not totally typical and the differential diagnosis includes NHL, for example if the immunophenotype score (see below) is 3 or less.

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

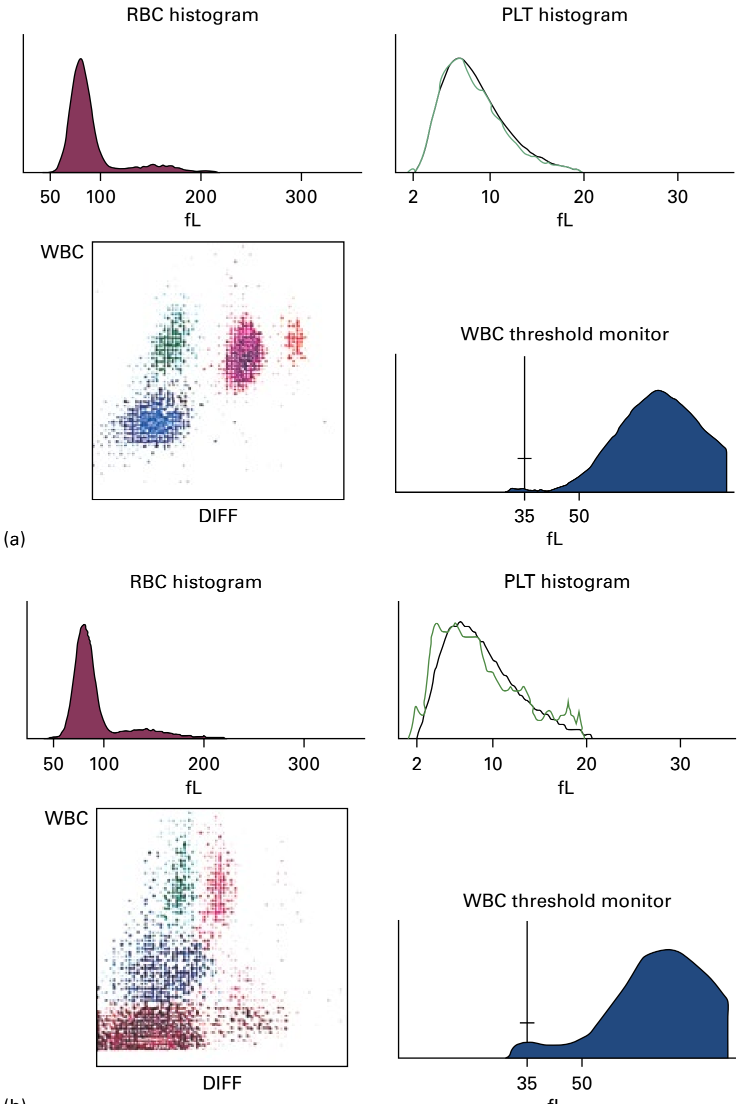


Fig. 7.9 Scatterplots on a Coulter Electronics Gen-S automated counter of PB from a patient with CLL (a) in comparison with the blood of a normal volunteer (b). The CLL sample shows an expanded lymphocyte cluster.

bone marrow may show increased mast cells (Fig. 7.10). Exceptionally, when a patient with CLL develops haemophagocytic lymphohistiocytosis, there is phagocytosis of lymphocytes as well as of myeloid cells [50].

CLL may undergo a prolymphocytoid transformation or a large cell transformation, referred to as Richter syndrome. In prolymphocytoid transformation both the blood (Fig. 7.11) 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. 7.12). The proliferation fraction may be high (Fig. 7.13). Richter syndrome represents transformation of a cell of the original clone in only two-thirds of patients and for

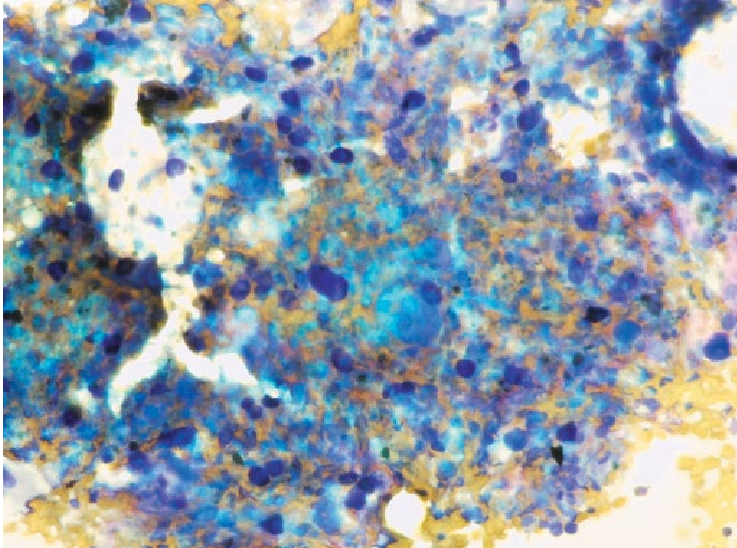


Fig. 7.10 Bone marrow aspirate in CLL showing a fragment containing numerous dispersed mast cells, which are stained deep purple. MGG ×40.

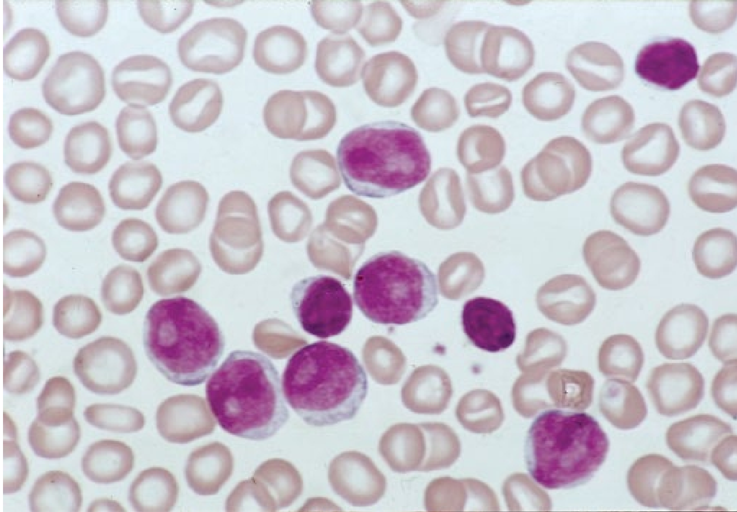
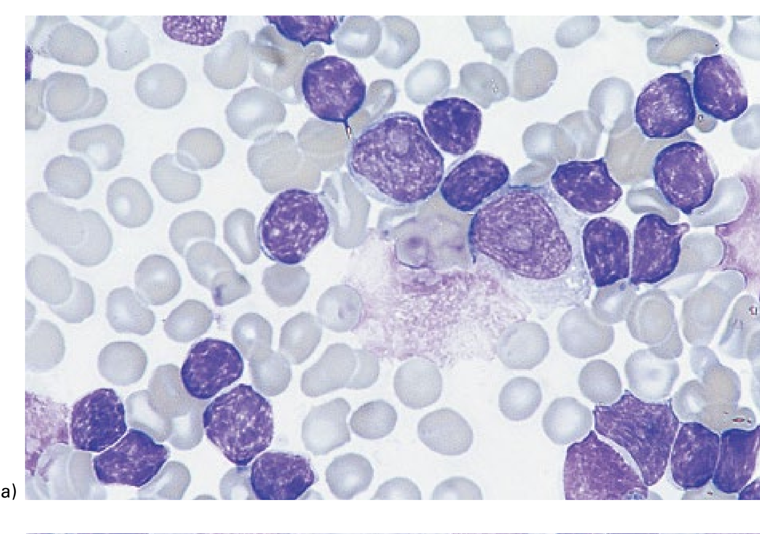


Fig. 7.11 PB film in prolymphocytoid transformation of CLL.

this reason the term 'Richter syndrome' may be preferred to 'Richter transformation'. 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 [51,52]. Fludarabine is suspected of being an aetiological factor [52]. Uncommon transformations are to Burkitt lymphoma or Hodgkin lymphoma. In some cases of Hodgkin lymphoma the Reed–Sternberg and Hodgkin cells appear to be derived from the CLL clone [52].

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 immunoglobulin expressed is usually IgM, with or without IgD.


(a)

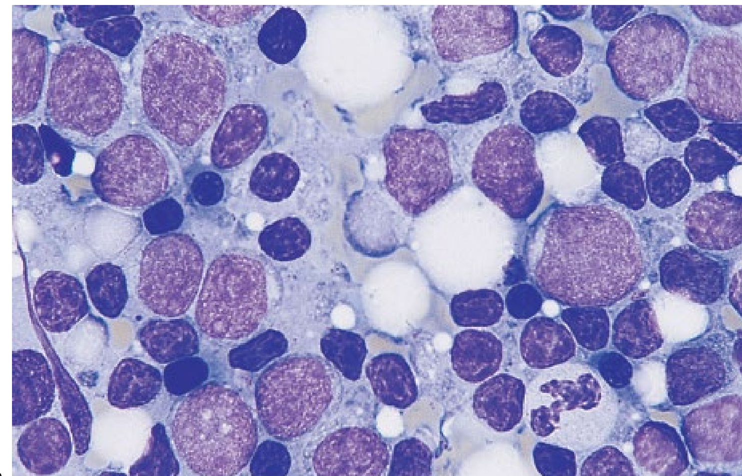

(b)

Fig. 7.12 (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.

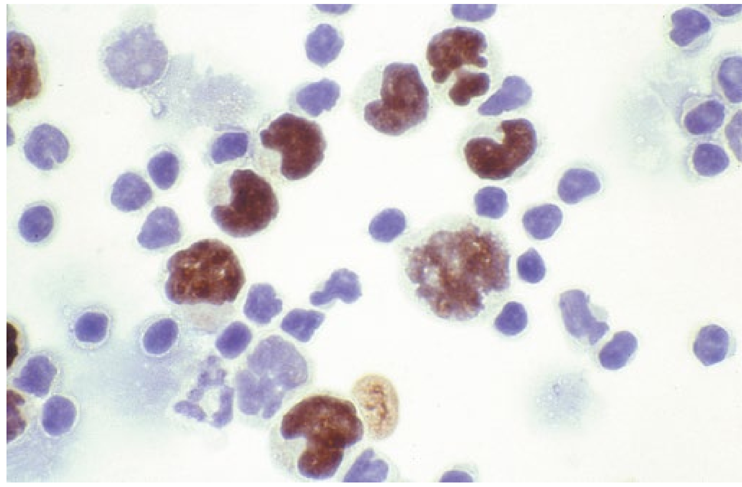


Fig. 7.13 Immunocytochemistry showing a high proliferative fraction in Richter syndrome; two of the large lymphoid cells are negative for the Ki-67 antigen and eight show strong nuclear staining. Immunocytochemistry for Ki-67.

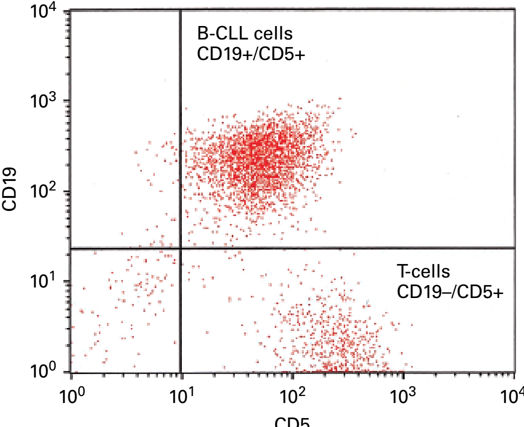


Fig. 7.14 Immunophenotyping by flow cytometry in CLL using a CD19 monoclonal antibody (McAb) labelled with phycoerythrin and a CD5 McAb labelled with fluorescein isothiocyanate; there is a major population of CD19+CD5+ CLL cells and a minor population of CD19–CD5+ normal T cells. (With thanks to Mr Ricardo Morilla, London.)

Cells are positive for B-cell-associated antigens such as CD19, CD20, CD24 and human leucocyte antigen DR (HLA-DR) (see Table 7.8). In addition, leukaemic cells of the great majority of patients express CD5 (also expressed by T cells) (Fig. 7.14), CD23 [53] and CD200. Expression of CD20 is usually heterogeneous and weak (weaker than expression of CD19) although bone marrow and lymph node cells show stronger expression than circulating cells [54]. FMC7 (detecting an epitope of CD20), CD22 and CD79b are usually absent or weak [55]; in three series of patients, CD79b was reported to be negative in 84%, 85% and 95% of cases respectively [56]. Surface membrane CD79a expression is reduced compared with normal B cells [57], the molecule being retained in the endoplasmic reticulum. CD200 is more strongly expressed than by normal B lymphocytes or the cells of other lymphoproliferative disorders (except hairy cell leukaemia) [58]; absence or quite weak expression is found in mantle cell lymphoma, which is potentially useful when there is diagnostic difficulty. CD200 expression is weaker when trisomy 12 is present [59]. The complement receptor C3bR (CD35), which is expressed on normal B cells, is usually absent from CLL cells [60]. In most series of patients, CD11c and CD25 have 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 in another CD25 was expressed in about a third of cases [61]; CD11c has been reported as positive in between 13% and 70% of patients in different series [62,63]; when positive, expression is weaker than in hairy cell leukaemia [63]. CD21, the C3d complement receptor and EBV receptor, is more weakly expressed than on normal B cells [60]. CD1c is expressed on about half of normal B cells, but is less often expressed in CLL [64]. CD45 is also often expressed more weakly than in other lymphoproliferative disorders and more weakly than by normal B and T lymphocytes. Low expression of CD45 correlates with increased leukaemic cell fragility, high numbers of smear cells, absence of trisomy 12, CD38 expression and better prognosis [33]. CD45RA is expressed. CD43 (used in immunohistochemistry) is expressed [65,66]; 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 [67]. 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 [68]. Strength of expression of CD52 is similar to that of normal lymphocytes [69], 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 [70]. CD49d is expressed in about 50% of cases [71]. Overexpression of *BCL2* protein, in comparison with expression on normal B lymphocytes,

can be demonstrated [65]. Nuclear cyclin D1 is expressed in a significant minority of patients [72]. Nuclear expression of Ki-67, a marker of proliferating cells, is very variable [73]. 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% [74]. 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% [74]. CD8 is expressed in a small minority of patients, 2% in one study [75,76]. LEF1 (lymphoid enhancer-binding factor 1), also expressed in T cells, is expressed in the nuclei of CLL cells but is not expressed in other small B-cell neoplasms [77].

A worse prognosis in CLL has been linked to expression of both IgM and IgD rather than expression of IgD alone [78], expression of CD25 [79,80], FMC7 [79], CD69 [67] and CD14 [79], high levels of expression of Ki-67 [73] and expression of cyclin D1 in more than 5% of cells [72]. Aberrant expression of myeloid or T-lymphocyte markers is also associated with a worse prognosis [74]. However, the most important immunophenotypic indicators of poor prognosis are ZAP70 expression [81,82] and CD38 expression [83–88]. 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 [78], lack of somatic hypermutation of *IGHV* (see below) [87], larger cells [89] and the development of autoimmune complications [87]. CD38 may be expressed on all cells or on a subpopulation of cells; either pattern of expression is associated with a poor prognosis [87]. ZAP70 expression similarly correlates with lack of somatic hypermutation and also with CD38 expression.

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 7.9) [53,55]. 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 and weaker expression of CD19 and CD20 in CLL, and the lack of expression of CD200 in mantle cell lymphoma [90]. In mantle cell lymphoma CD19 is more weakly expressed than CD20 whereas the reverse is true in CLL [91].

Table 7.9 A scoring system for the immunophenotypic diagnosis of chronic lymphocytic leukaemia (CLL) [53,55].

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

SmIg, surface membrane immunoglobulin.
* Or alternatively CD22 negative but discrimination is better if CD79b is used [55].

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 [92]. In addition, normal individuals have, on average, about 12% of CD5-positive B cells in the peripheral blood [93]; 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−) [94].

In Richter syndrome the immunophenotype of CLL is generally retained although there may be altered expression of one or more antigens [51]. For example, CD5 expression may be lost. It seems likely that whether or not the immunophenotype is that of CLL is related at least in part 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 that 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 diffuse 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. They are composed of lymphocytes, prolymphocytes, paraimmunoblasts and follicular dendritic cells.

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 pseudofollicular pattern. Occasionally nodal infiltration is partial with an interfollicular or perifollicular pattern [15]. The spleen shows variable infiltration of red and white pulp; white pulp infiltration usually predominates [15]. The infiltrate in the white pulp may show a pseudofollicular pattern, attributable to the presence of proliferation centres [15].

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 [15]. Nuclear expression of LEF1, which is not seen in normal mature B cells, can help in making a distinction from NHL of small B cells [95].

Cytogenetic and molecular genetic features

Cytogenetic abnormalities [15,96–98] 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. 7.15) 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; in one study various balanced and unbalanced translocations were detected in about a third of cases, their presence being associated with worse prognosis [99], and in another a cytogenetic abnormality was detected in more than 80% of patients [100].

The most characteristic abnormalities are deletion or rearrangements with a 13q14.3 breakpoint (60–80% of cases) and del(11)(q22-23) (10–20% of cases) [101]. A study of 353 patients found deletion of 13q14 in 61% of patients [102]. In the same large study, deletion of 11q22.3 was found in 27% [102]. Less common cytogenetic abnormalities are trisomy 12 (10–20% of cases) or partial trisomy of 12q13, del(17)(p13) (5% of cases at diagnosis but 50% or more with advanced disease), del(6)(q21) (5–10% of cases), duplication of 2p, 8q and 15q, translocations with a 14q32 breakpoint (4–9% of cases) and other translocations. Some patients have complex karyotypic abnormalities, which are of adverse prognostic significance [103]. There is a negative correlation between the presence of trisomy 12 and the presence of 13q14.3 abnormalities suggesting that these karyotypic

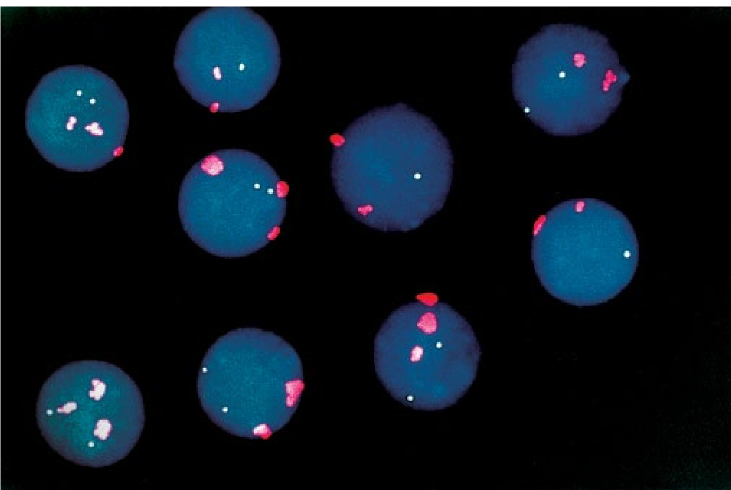


Fig. 7.15 Fluorescence *in situ* hybridization (FISH) in CLL using directly labelled probes for chromosome 12 (pink) and *RB1* (white). In this patient there were three subclones within the population of CLL cells: (i) cells that are disomic for chromosome 12 and have both alleles of *RB1* (two pink dots and two white dots); (ii) cells that are disomic for chromosome 12 but have a deletion of one allele of *RB1* (two pink dots and one white dot); and (iii) cells that have trisomy 12 and no *RB1* deletion (three pink dots and two white dots). (With thanks to Dr J. Garcia Marco, Cambridge.)

abnormalities are associated with two independent leukaemogenic mechanisms. In some studies, trisomy 12 has been found to correlate with atypical morphology and a worse prognosis [104,105], 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 [39,106,107]. The good prognosis associated with loss of 13q14 is confined to cases that do not have the deletion in the great majority (>80%) of cells [108]. 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 [96,109]. 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 [110]. The presence of both del(17)(p13.1) and del(11)(q22.3) in a single clone is associated with a worse prognosis than either alone [111]. Del(14q) was found in 1.9% of a large series of patients and was prognostically adverse [112]. In the UK Leukaemia Research Fund CLL 4 trial, best prognosis 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 these were statistically significant [113]. 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)(p11.2;q21) and t(18;22)(q21;q11.2) are more common [97]; the breakpoints on chromosome 18 differ at a molecular level from the breakpoints in follicular lymphoma [114]. The presence of t(14;18) correlates with trisomy 12 (13/22 cases) and mutated *IGHV* genes (20/21) [115]. A t(14;19)(q32.3;q13.3) translocation is found in less than 1% of patients, the genes involved being the IGH locus and *BCL3* [116]; these patients often have atypical features including relatively young age, atypical cytology and immunophenotype, and probably a worse prognosis [117,118]; however, if the cytology and immunophenotype are typical of CLL, the prognosis is not adverse [118]. There is an association of t(14;19) with trisomy 12 [118]. Rearrangements at 14q32 are also associated with a poor prognosis

[119]. *MYC* translocation associated with t(8;14)(q24.2;q32.3), t(8;22)(q24.2;q11.2) or t(2;8)(p11.2;q24.2) is associated with increased prolymphocytes and poor prognosis [120]. An unbalanced rearrangement, dic(17;18)(p11.2;p11.2), found in 1.3% of a very large series of patients, is usually associated with a complex karyotype and with early age of diagnosis and accelerated disease progression [121]. There have been reports of t(11;14)(q13.3;q32.3) and *CCND1* rearrangement in a small proportion of patients with CLL, but it is quite likely that this represents misdiagnosis of mantle cell lymphoma. A small minority of patients have a jumping translocation, often involving 17p11.2 and associated with loss of *TP53* and an adverse prognosis [122].

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 [123]. They are more likely to have strong expression of CD20 and to respond to rituximab [124]. Cases with a complex karyotype are also more likely to express FMC7 [123]. Cases with del(11q) have been found to have reduced expression of numerous adhesion molecules, including CD11a/CD18, CD11c/CD18, CD48 and CD58 [125]; expression of CD20 is also low and may indicate lower probability of response to rituximab [124].

Molecular genetic analysis has shown that CLL can arise either from a mutation in a B cell with non-mutated *IGHV* genes or from a post-germinal centre memory B cell with hypermutated *IGHV* 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 [15,86,126,127]. The differences observed in these two subsets of CLL are summarized in Table 7.10 [15,128–134]. Origin from a cell with non-mutated *IGHV* genes has been reported to correlate well with

Table 7.10 Differences between chronic lymphocytic leukaemia subsets with unmutated and hypermutated immunoglobulin heavy chain variable region (*IGHV*) genes [15,128–134].

	Somatic hypermutation of *IGHV* genes absent (>98% homology with germline)	**Somatic hypermutation of *IGHV* genes present (≤2% homology with germline)**
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 [128]; CD180 less strongly expressed [129]; IgM more strongly expressed [129]	CD38 not usually expressed; *MUM1-IRF4* expressed [128]; CD180 more strongly expressed [129]; IgM less strongly expressed [129]
Cytogenetics	Increased prevalence of abnormalities of 11q and 17q and, in some studies, of trisomy 12 [15]	Increased prevalence of 13q14.3 abnormalities [15]
Molecular genetics	*TP53* dysfunction (due to *TP53* or *ATM* mutation) more common; *ZAP70* expression [130]; *CLLU1* markedly upregulated [131]; mutation of *MYD88*, *CHD2* and *KLH6* more likely [132]	*TP53* dysfunction less common; lack of *ZAP70* expression [130]; *CLLU1* moderately upregulated [131]; *BCL6* may be mutated and correlates with worse prognosis [133]; mutation of *NOTCH1*, *TP53*, *SF3B1*, *POT2* and *XPO1* more likely [132]
Histology	Proliferation centres absent in trephine biopsy sections [134]	Proliferation centres may be present in trephine biopsy sections
Prognosis	Worse	Better

CD38 expression [84,87,134] although some observers have reported poorer correlation [83,86,135]. Unmutated genes and CD38 expression have been found to be independent poor prognostic features [135]. ZAP70 expression also correlates with unmutated genes and indicates a worse prognosis [81]. In one study ZAP70 was a better surrogate marker for unmutated genes than CD38 [81], 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 [81]. ZAP70 testing is now less often performed as there are other relevant markers that are easier to test for. The presence of *BCL6* mutation identifies a poorer prognosis subset within the groups of patients with hypermutation of *IGHV* [133].

At a molecular level, one study of abnormalities of chromosome 13 [136] 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, *MIR16-1* and *MIR15A* [15]. Deletion of 13q14 leads to deletion of *DLEU1* and *DLEU2* [102]. *ATM* at 11q23 is deleted in patients with del(11q) and overall is mutated in about 20% of patients [137]; mutations are seen particularly in patients in whom the other allele is deleted [138]. Deletion of 11q22-23 can lead to loss of *ATM* and *BIRC3* as well as two genes encoding microRNAs, *MIR34B* and *MIR34C* [101,102]. The *BCL6* gene was mutated in about a quarter of patients in one study [139]. The leukaemogenic mutation associated with trisomy 12 has not yet been elucidated but amplification of the *MDM2* gene at 12q15 has been suggested as a possible mechanism [98]. 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 [140]. 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 the reduced surface expression of CD79b and IgM [141]. *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 [138]. Overall, a *TP53* mutation or deletion is found in about 15% of patients but is relatively uncommon in typical CLL, correlating with progressive disease, refractoriness to therapy and poor prognosis [142]. Strong expression of TP53 correlates with worse prognosis [143]. Investigation of *TP53* is particularly relevant in young patients who might benefit from intensive treatment including stem cell transplantation. In some patients, dysfunction of TP53 is the result of *ATM* mutation, probably biallelic loss or mutation [144]. Mutation of *ATM* is found in about 9–12% of patients and correlates with refractoriness to chlorambucil and fludarabine and with worse survival, independent of *IGHV* status [145,146]. More than 30 genes have been reported to be mutated in CLL. Those mutated in 10% or more of patients include *SF3B1*, *ATM*, *TP53*, *CHD2*, *NOTCH1*, *MYD88*, *POT1*, *NFKBIE* and *FAT1* [101,147]. Mutations of *BIRC3* are also relatively frequent [15]. In three series of patients, *NOTCH 1* was mutated in 4–12% of patients, *MYD88* in 3–10% and *SF3B1* in 15–18.4% [146,148,149]. The prevalence of various cytogenetic abnormalities and loss of specific cancer suppressor genes can be related to whether or not *IGHV* shows hypermutation (see Table 7.14). Although *BCL2* is often overexpressed, rearrangement is demonstrable in only a small minority of patients [97]. Other recurrent molecular abnormalities include mutations of *XPO1* (*c.* 2%) and *KLHL6* (*c.* 2%) [148]. *NOTCH1* and *XPO1* mutations are mainly associated with unmutated *IGHV*; *NOTCH1* mutations correlate with a worse prognosis [148]. *MYD88* and *KLHL1* mutations mainly occur in association with mutated *IGHV* [148]. *FBXW7* may also be mutated.

Table 7.11 The Rai staging system for chronic lymphocytic leukaemia.

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 $<100 \times 10^9$/l)*

Hb, haemoglobin concentration.
* Anaemia or thrombocytopenia with an immune basis also lead to categorization as stage III or IV disease [152].
Derived from reference 151.

Table 7.12 The Binet staging system for chronic lymphocytic leukaemia*.

Stage	Prognosis	Criteria
A	Favourable	Lymphocytosis with no more than two regions† having enlargement of lymph nodes or other lymphoid organ; Hb >100 g/l and platelet count $>100 \times 10^9$/l
B	Intermediate	Lymphocytosis with enlargement of lymph nodes or other lymphoid organ in three or more regions; Hb >100 g/l and platelet count $>100 \times 10^9$/l‡
C	Unfavourable	Hb <100 g/l, platelet count $<100 \times 10^9$/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 [152].
Derived from reference 152.

When Richter syndrome represents transformation of the original clone there is cytogenetic and molecular evolution. In one study of 43 patients, three (7%) were found to have a *BRAF* V600E mutation, an observation of possible relevance to therapy since a BRAF inhibitor is available [150].

Prognosis

Prognosis is related to patient and disease characteristics and the stage of the disease. The Rai [151,152] and Binet [153] staging systems for CLL incorporate clinical and haematological features (Tables 7.11 and 7.12). It is also possible to combine these two systems (see footnote to Table 7.12). In addition, it may be useful to recognize patients with 'smouldering chronic lymphocytic leukaemia' (Table 7.13) in whom the disease is likely to run a very indolent course. UK Leukaemia Research Fund trials showed that progressive stage A disease (e.g. lymphocyte doubling time less than 12 months, falling haemoglobin concentration (Hb) and falling platelet count) had the same survival as stage B disease and could therefore be grouped with it. It should be

Table 7.13 Criteria for a diagnosis of smouldering chronic lymphocytic leukaemia.

Criteria
Binet stage A
Non-diffuse pattern of bone marrow infiltration
Haemoglobin concentration >130 g/l
Lymphocyte count $<30 \times 10^9$/l
Lymphocyte doubling time >12 months

noted that patients assigned to an advanced stage on the basis of cytopenia that is autoimmune in nature have a better prognosis than those whose cytopenia results from heavy bone marrow infiltration [43]. The Rai and Binet staging systems retain relevance when facilities for molecular and biochemical analyses are not available.

Factors that have prognostic significance are summarized in Table 7.14 [71,132,154–168].

Various analyses have sought to identify the most significant variables. In a study of 339 patients with Binet stage A disease it was found that, on multivariate analysis, prognosis could be predicted from serum thymidine kinase more than 10 iu/l, a lymphocyte count of more than 13×10^9/l, β2 microglobulin more than 2.5 mg/l and CD38 expression on more than 7% of cells [156]; in this study no extra prognostic

Table 7.14 Factors indicating worse prognosis in chronic lymphocytic leukaemia [71,132,154–168].

Biological features
Older age (e.g. >60 years)
Male gender*
Worse performance status
Features directly or indirectly indicative of stage and aggressiveness of disease
Anaemia (e.g. Hb <100 g/l) [154]
Thrombocytopenia (e.g. platelet count $<100 \times 10^9$/l) [154]
Absolute lymphocyte count [155], e.g. $>30 \times 10^9$/l or $>50 \times 10^9$/l
Doubling time of lymphocyte count <12 months in Binet stage A patient
Advanced stage disease (Rai or Binet)
Number of involved lymph node groups† [155]
B symptoms [154]
Bone marrow showing diffuse infiltration (‘packed marrow’ pattern)
Higher soluble CD23 or CD138 in serum
Higher β2 microglobulin (e.g. >2.5 mg/l) [156]
Higher lactate dehydrogenase (e.g. >210 iu/l)
Higher serum thymidine kinase (10–23.9 or >24 u/l) [156]
Higher plasma thrombopoietin [157]
Higher serum chemokines, CCL3 and CCL4 [132]
Features of leukaemic cells and response to leukaemic infiltration
Smear cells <30%
Prolymphocytes in blood
Enhanced bone marrow angiogenesis and higher blood VEGF levels [158]
More numerous mast cells [159]
Higher ZAP70 expression (e.g. 20% of cells or more)‡ or higher CD38 expression (e.g. 20% or 30% of cells or more)‡ or both [160]
Higher expression of CD49d (>45% of cells [161] or ≥30% of cells [71])
Higher expression of CD1d [162]
Abnormal serum free light chain ratio [163]

(*Continued*)

Table 7.14 (Continued)

Expression of lipoprotein lipase
Expression of CLLU1
Unmutated *IGHV*[‡ §] [164] or use of VH3-21 whether *IGHV* is hypermutated or not [165]; use of VH3-21 or VH3-23 rather than VH3-73 or VH3-30 (known as stereotypy) [166]
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, *BIRC3* abnormality [167]
NOTCH1 and *SF3B1*mutation (intermediate or adverse) (*MYD88* mutation is favourable) [132]
NFKBIE, *RPS15* or *IKZF3* mutation [132]
−938C → A polymorphism in promoter of *BCL2* gene [168]

Hb, haemoglobin concentration; 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*.

[†] Adds extra information to stage.

[‡] Interrelated but independent prognostic factors; ZAP70 expression also correlates with higher stage disease, higher β2 microglobulin and more rapid lymphocyte doubling time [160].

[§] Unmutated is often defined as ≥98% homology with germline; such cases have a worse prognosis than cases showing 97–98% homology with germline, which, in turn, have a worse prognosis than cases with <97% homology [164].

information was derived from *IGHV* mutational status, ZAP70 status or common cytogenetic abnormalities, del(13q), +12, del(11q) and del(17p) [156]. In another study CD49d expression (with at least 30% of cells being positive) was the most significant immunophenotypic marker for overall survival and treatment-free survival, with CD38 and ZAP70 expression adding little extra prognostic information [71]; on multivariate analysis the only other factors predictive of overall survival were age, gender, *IGHV* mutation status, del(17p) and the absolute lymphocyte count.

In a further study of 1154 patients with Binet stage A disease, independent prognostic features were lymphocyte doubling time, *IGHV* mutation status, CD38 expression and age at diagnosis [169]. A further study proposed a prognostic model based on age, gender and serum β2 microglobulin [155]. *NOTCH1* [149] and *SF3B1* mutations [146,149] are independent adverse prognostic factors. In a study of 1274 patients, molecular analysis was found to add prognostic information to that obtained from cytogenetic analysis [167]. Patients could be assigned to four groups with 5-year survival ranging from 37% to 69%: high risk, *TP53* and/or *BIRC3* abnormality; intermediate risk, *NOTCH1* and/or *SF3B1* mutation and/or del(11)(q22-23); low risk, trisomy 12 or normal cytogenetics; very low risk, del(13)(q14) only [167]. In a further comprehensive study, 23 prognostic markers were assessed in 1948 patients [154]. The independent predictors of overall survival were: gender (better survival in women), age, ECOG (Eastern Cooperative Oncology Group) performance status, del(17p), del(11q), *IGHV* mutation status, β2 microglobulin and serum thymidine kinase [154]; these can be combined to give a prognostic score dividing patients into four risk groups with 5-year survival varying from 19% to 95% [154].

The presence of 10% or more prolymphocytes correlates with the presence of *NOTCH1* mutations, absence of 13q deletion, high CD38 expression, unmutated *IGHV* genes, probability of Richter syndrome and worse progression-free and overall survival [35].

It has been suggested that, when resources permit, *IGHV* status and FISH for relevant abnormalities should be performed on all patients [170].

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.

Some cases of CLL have cytologically atypical features and are thus more likely to be confused with NHL. The French–American–British (FAB) group defined two morphological variants, 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% 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) [171,172] (Fig. 7.16). Cytologically atypical cases tend to have a worse prognosis [39,173,174] and a different frequency of various cytogenetic and molecular genetic abnormalities [112,127,136,142]. In the WHO classification cytologically atypical cases are not distinguished.

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 *IGHV* genes) in the peripheral blood [3,175–179]. B-cell numbers may 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% [176]. In about three-quarters of such cases the immunophenotype resembled that of CLL and in the other quarter, that of NHL [176].

In cases with the immunophenotypic features of CLL, clonal cytogenetic abnormalities typical of CLL are often present, 13q14 abnormalities being found in 39% and trisomy 12 in 18% in one series of patients [176]; del(6q) may also be observed [178]. The poor prognosis abnormalities, del(11)(q23) and del(17)(p13), are less frequent than in CLL [176,178]. Somatic *IGHV*

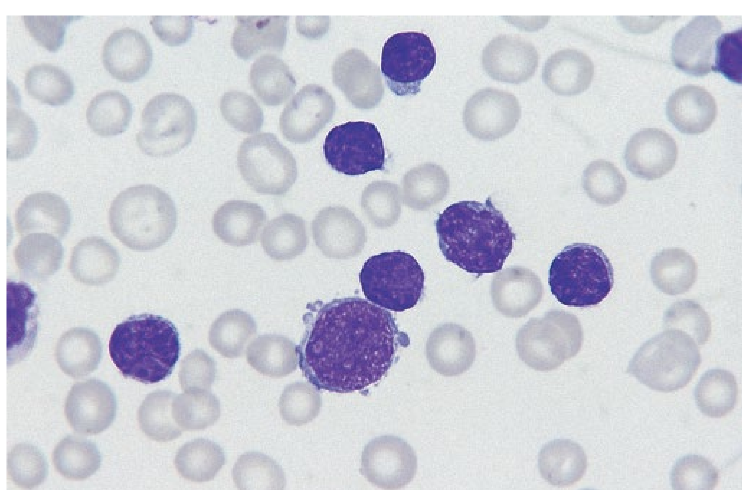


Fig. 7.16 PB film in CLL, mixed cell type, showing small mature lymphocytes and several larger cells, one with multiple nucleoli. MGG ×100.

hypermutation, a good prognosis feature in CLL, was present in 88% of patients in one series [176] and 76% in another [178]. CD38 expression, also a marker of indolent CLL, was usually low in one series [176] but was significantly more often positive in another [178]. Some patients with monoclonal B-cell lymphocytosis progress to CLL. 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 [176]. A shorter time to progression requiring treatment is predicted by ZAP70 expression, the presence of IGH locus translocations, the presence of del(11)(q22.3) and the absence of somatic mutation of *IGHV*, with only unmutated *IGHV* being predictive on multivariate analysis [178]. Progression also is more likely if there are other adverse prognostic abnormalities or at least 0.5×10^9/l clonal B cells [179]. Patients who present with CLL have usually, probably always, had preceding monoclonal B-cell lymphocytosis [16].

Suggested diagnostic criteria are shown in Table 7.15 [177,179].

The prevalence of monoclonal B-cell lymphocytosis is higher (about 13%) in first-degree relatives of patients with CLL [180]. It may be relevant to identify this condition when a relative is being assessed as a potential donor for haemopoietic stem cell transplantation [181].

A minority of cases have an atypical CLL immunophenotype or a non-CLL immunophenotype. A proportion of such cases show progression to NHL.

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 [182].

Table 7.15 Criteria proposed for the diagnosis of monoclonal B-cell lymphocytosis [177,179].

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 [177] or is less than 5×10^9/l [179] and there are no features diagnostic of a B-lineage lymphoproliferative disorder (other than possibly a paraprotein)

Clinical, haematological and cytological features

There is a higher median age of onset than in CLL. Characteristically there is a high white cell count (WBC) and marked splenomegaly with only trivial lymphadenopathy [183].

The predominant cell is the prolymphocyte (Figs 7.17 and 7.18); a cut-off point of 55% of such cells has been found to be most useful in separating prolymphocytic leukaemia from CLL/PL [172]. 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. Occasionally the cytoplasm contains globules, Auer rod-like crystals [184] or azurophilic granules [185], which are composed of immunoglobulin. One patient whose cells showed erythrophagocytic activity has been described [186].

Anaemia and a high lymphocyte count have been found to correlate with a worse prognosis [187]. A positive direct antiglobulin test and AIHA may occur [187]. There may also be thrombocytopenia. The serum immunoglobulin concentration is often reduced and a paraprotein is present in about a third of patients [187].

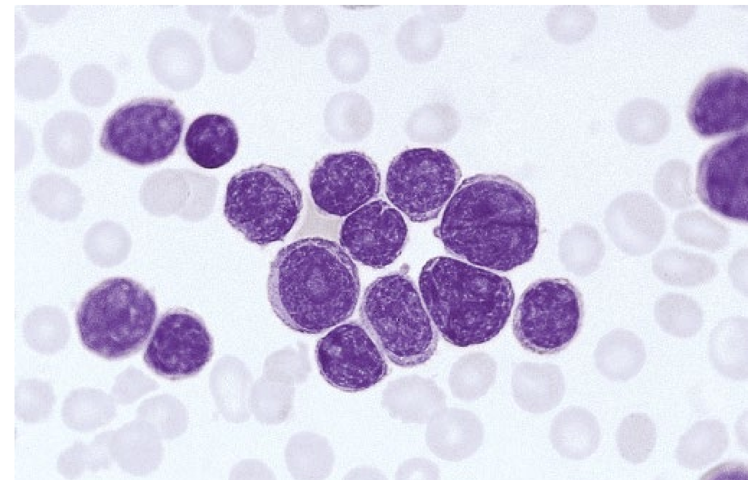


Fig. 7.17 PB film in B prolymphocytic leukaemia (B-PLL) showing cells that are regular in shape with round nuclei. Nuclear chromatin shows some condensation and the larger cells contain prominent vesicular nucleoli. MGG ×100.

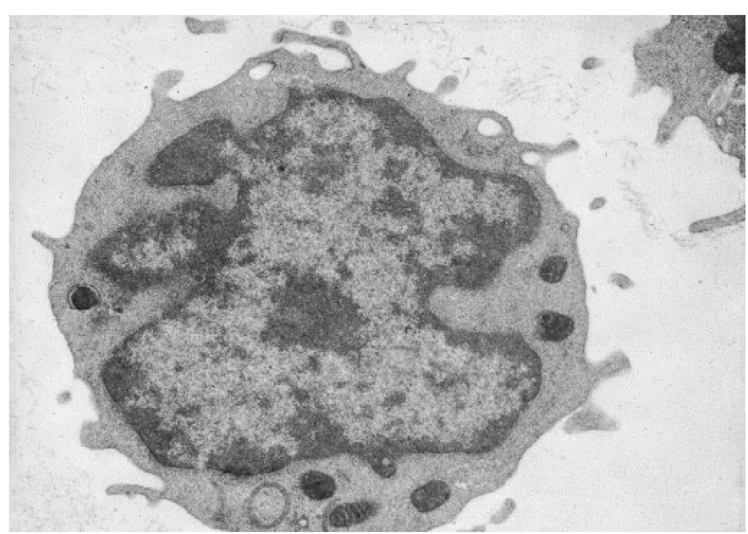


Fig. 7.18 Ultrastructural examination in B-PLL showing a prominent nucleolus and abundant cytoplasmic organelles. (With thanks to Professor Daniel Catovsky, London.)

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 majority of cases of PLL there is expression of IgM with or without IgD, but in a minority there is expression of IgG or IgA. In the other third of cases, the immunophenotype is intermediate between the typical CLL phenotype and the typical PLL phenotype. CD19, CD22 [188], CD79a and CD79b [189] are expressed. CD5 is expressed in 20–30% of cases and CD23 in 10–20% [190]. CD200 is usually weak or not expressed [191]. CD11c is strongly expressed in the majority of patients [63]. In some patients, leukaemic cells express CD5 and show expression of FMC7 and strong expression of SmIg, thus immunophenotypically resembling cells of mantle cell lymphoma [192]. In one series of patients, CD10 and CD38 were often expressed [187]. ZAP70 is often expressed [182].

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 and without proliferation centres. 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 [193,194].

Cytogenetic and molecular genetic analysis

There are often complex karyotypic abnormalities. Abnormalities that have been noted include trisomy 3 [188], del(6q), monosomy 7, del(7q), del(11)(q23), del(12)(p13), del(13)(q14), del(17)(p13) and various translocations involving chromosome 14 with a 14q32 breakpoint [188,195–197]. There may be *MYC* amplification or t(8;14)(q24.1;q32) with *MYC* brought into proximity to the IGH locus [198]. Other translocations observed have included t(6;12)(q15;p13), t(2;3)(q35;q14) [195] and t(3;8)(p13;q13) [199]. Trisomy 12 occurs [195] but is relatively uncommon [182].

It should be noted that previously described cases associated with t(11;14)(q13.3;q32) are now considered to represent a leukaemic phase of mantle cell lymphoma [182].

Hypermutated and non-mutated *IGHV* genes are equally frequent but the mutational status shows no relationship to CD38 or ZAP70 expression [182]. Mutations of *TP53* [200] and deletion of *RB1* [197] 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. Nuclear expression of cyclin D1 or *SOX11* is indicative of a diagnosis of mantle cell lymphoma rather than B-PLL [201]. 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 [202,203].

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 [204]. The US incidence is 0.32/100 000/year [204]. The disease is characterized by splenomegaly with little peripheral lymphadenopathy. Using computerized tomography (CT) scanning, abdominal or mediastinal lymphadenopathy is sometimes detected at diagnosis, mainly in patients with bulky disease. Occasionally there is involvement of the liver or bones (bone lesions may be lytic). Rarely there are pleural effusions or ascites, or involvement of the gastrointestinal tract or central nervous system [205]. 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 [206,207]. 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

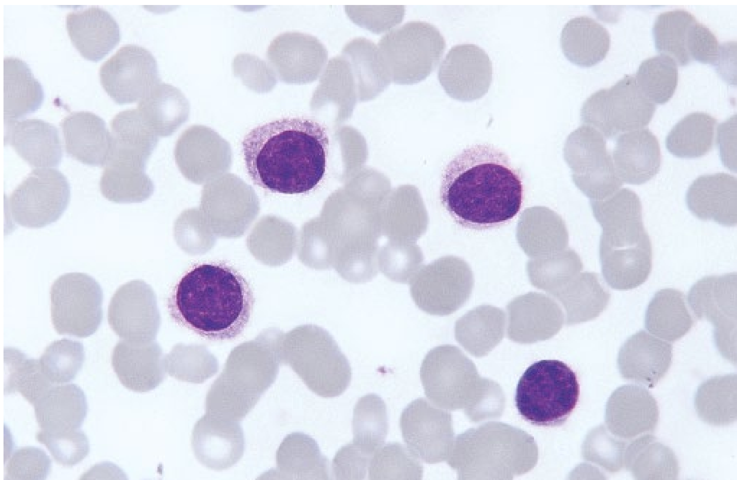


Fig. 7.19 PB film in hairy cell leukaemia. Cells have round nuclei with condensed chromatin and moderately abundant cytoplasm with ragged edges. MGG ×100.

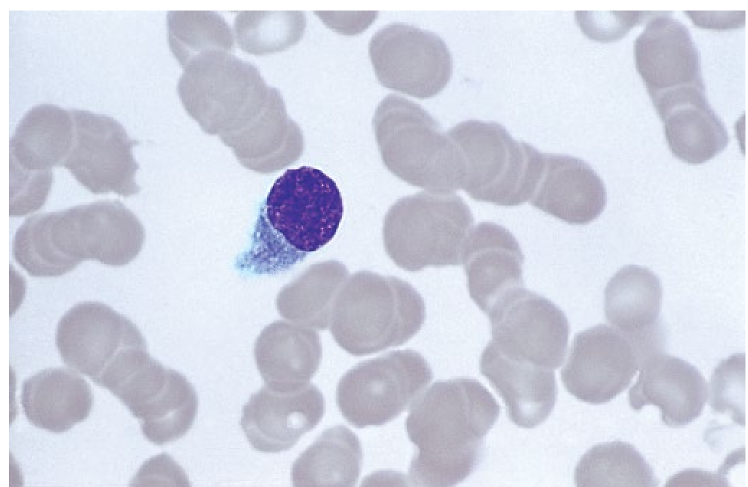


Fig. 7.20 PB film in hairy cell leukaemia showing a hairy cell containing a ribosomal lamellar complex. MGG ×100. (With thanks to Dr Laura Sainati, Padua, and Professor Daniel Catovsky.)

and consequently an ill-defined cell outline (Fig. 7.19). The cytoplasm may contain azurophilic granules or rod-shaped inclusions. Occasionally there are parallel linear structures in the cytoplasm (Fig. 7.20) that correspond to the ribosomal lamellar complex that is demonstrated on ultrastructural examination. The nucleus is eccentric, and round, oval, dumbbell- or kidney-shaped. Occasional hairy cells in some patients have ring, horseshoe-shaped or lobulated nuclei or are binucleated [208]. 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. 7.21). 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.

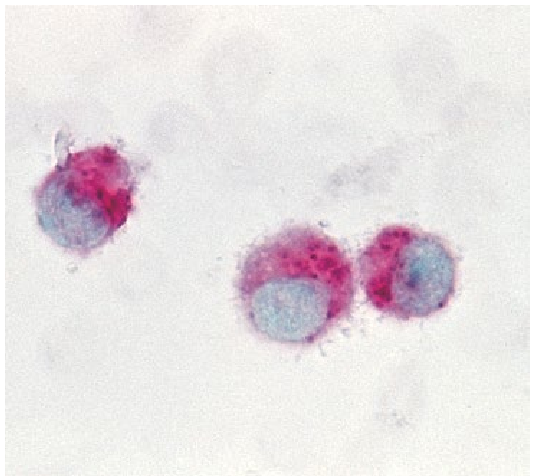


Fig. 7.21 Film prepared from a buffy coat of the PB of a patient with hairy cell leukaemia showing tartrate-resistant acid phosphatase (TRAP) activity. TRAP reaction ×100.

Immunophenotype

Hairy cells have distinctive light-scattering characteristics on flow cytometry [192,209]; forward light scatter is usually higher than in other chronic lymphoproliferative disorders, and side 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 [91]. Non-specific binding is common so that comparison with the negative control is important. Autofluorescence can occur, again giving importance to comparison with the negative control [190].

Hairy cells have the immunophenotype of a relatively mature B cell. B-lineage-associated antigens CD19, CD20, CD22 and CD79a are expressed. The expression of CD20 and CD22 is strong. CD79b is positive in about a quarter of patients [189]. 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 [202]. CD5, CD10, CD23 and CD43 are generally negative, with both CD10 and CD23 being expressed in about 10–15% of cases [210,211]. 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 [91]. 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 and CD123. In one study CD103 was expressed in all of 114 cases of hairy cell leukaemia and in 20/20 cases of hairy cell variant leukaemia, but was negative in all cases of mantle cell lymphoma (21 cases), follicular lymphoma (three cases), chronic lymphocytic leukaemia (133 cases) and splenic marginal zone lymphoma (nine cases) [212]. In the same study CD123 was expressed in all cases of hairy cell leukaemia but was more widely expressed: partial or weak in 8/20 hairy cell variant; and positive in 1/4 splenic marginal zone lymphoma, 7/21 mantle cell lymphoma, 1/3 follicular lymphoma and 5/133 CLL [212]. CD160 is expressed; otherwise this antigen is expressed by subsets of T and NK cells and, among B-cell neoplasms, typically only by CLL cells and then more weakly [213]. CD27 is not expressed whereas it is usually expressed in CLL, follicular lymphoma, mantle cell lymphoma and splenic lymphoma with villous lymphocytes [214]. CD200 is overexpressed in comparison with normal B cells [215]. 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 CD11c, CD25, CD103 and CD123 (replacing HC2) 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 [216].

Flow cytometry is a very sensitive technique for the detection of hairy cells and as few as 1%

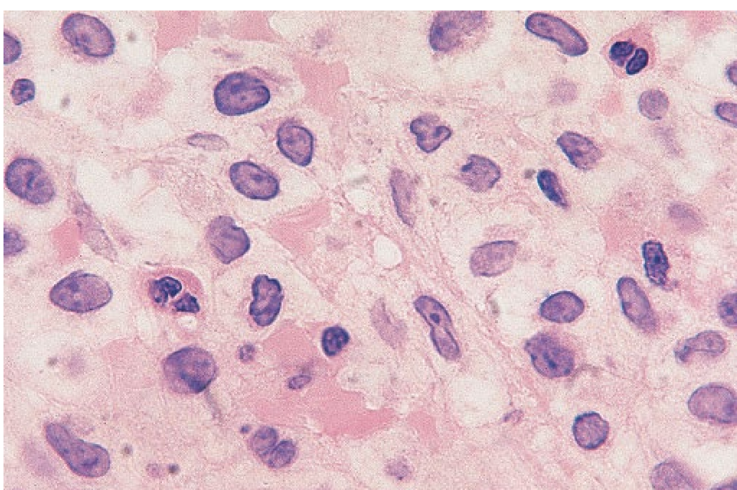


Fig. 7.22 BM trephine biopsy section in hairy cell leukaemia showing cells with round, oval or irregular nuclei and scanty, ragged cytoplasm. The spacing of the nuclei is characteristic of this disorder. Two neutrophils and one erythroblast are also present. Paraffin-embedded, haematoxylin and eosin (H&E) ×100.

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 [217].

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. 7.22). 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. There may be disruption of the microvasculature, leading to extravasation of red cells. Reticulin is increased. Osteosclerosis is occasionally observed [218]. 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 CD72 (DBA.44) [219]. Expression of annexin A1 is highly specific as long as it is clear that it 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 *CCND1* rearrangement [203]. 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. There is also an antibody available that is specific for the BRAF V600E protein (see below) [220]; this can be useful for MRD monitoring. Strong expression of T-bet has a high degree of specificity for HCL and can also be used for MRD monitoring [221].

Cytogenetic and molecular genetic analysis

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)) [125,222,223]. *BRAF* mutation is strongly associated with hairy cell leukaemia. In the first series of patients reported, the *BRAF* V600E mutation was found in all

48 patients with hairy cell leukaemia and had a high degree of specificity for this disease among B-cell neoplasms [224]; in another series of patients mutation was found in 42 of 53 patients (79%) with the mutation being absent when there was expression of IGHV4-34 [225]. Mutations are generally heterozygous but sometimes homozygous [224]. An alternative mutation, *BRAF* F468C or *BRAF* D449E, is present in a minority of patients [226]. Allele-specific PCR for detection of *BRAF* V600E is useful in the diagnosis of hairy cell leukaemia [227]. The second most commonly mutated gene is *CDKN1B*, an inactivating mutation being found in 16% of 81 patients in association with *BRAF* mutation [228]. BRAF-unmutated cases may have mutation of *MAP2K1*. Hairy cell leukaemia is usually associated with hypermutated *IGHV* genes; the minority of patients with unmutated *IGHV* have a more aggressive disease and are refractory to cladribine [229].

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 leukaemia variant (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 markedly hypocellular marrow. As long as the possibility of HCL is considered the diagnosis can be made without difficulty.

Hairy cell leukaemia variant

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 2016 revision of the WHO classification it is one of two provisional entities designated splenic B-cell lymphoma/leukaemia, unclassifiable. Hairy cell variant differs from HCL immunophenotypically, histologically and in its responsiveness to therapy [230].

Clinical, haematological and cytological features

There is usually splenomegaly with little lymphadenopathy. The WBC is usually high with peripheral blood leukaemic cells being numerous [231,232]. 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 usually has a more condensed chromatin pattern with a prominent nucleolus (Fig. 7.23). The nucleus resembles that of a prolymphocyte. There may be some binucleated cells and some larger cells with hyperchromatic nuclei. Occasionally nuclei have more dispersed chromatin or are convoluted [230]. 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. SmIg is strong [230]. CD25 is usually negative. CD11c and CD103 may be positive [230]. In one study CD103 was positive in 20/20 cases [212]. CD123 has been reported to be negative [199] but in another study 8/20 cases showed weak or partial expression [212]. CD79b is positive in about a quarter to a third of patients [189]. Although the α chain of the interleukin 2 receptor (CD25) is not expressed, there is expression of the β and γ

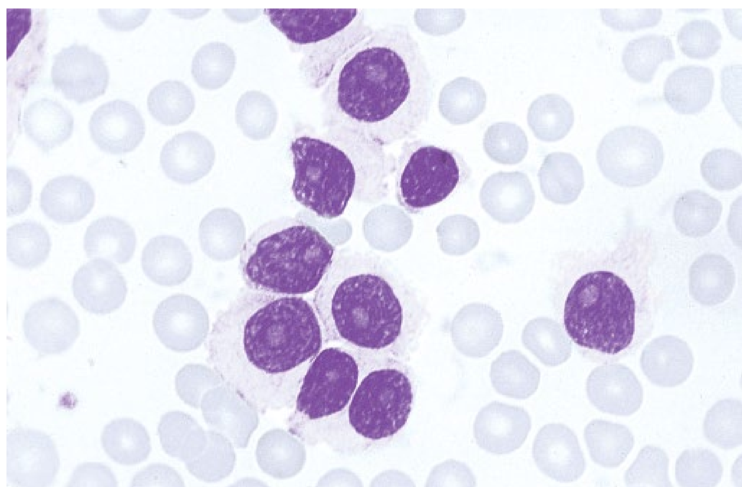


Fig. 7.23 PB film in the variant form of hairy cell leukaemia (hairy cell leukaemia variant). Cells are nucleolated and it is evident that the white cell count is high. MGG ×100.

chains [199]. CD200 is usually negative [230]. A scoring scheme for immunophenotypic markers has been found very useful if four McAb are used [216], CD123 now replacing HC2.

Histology

Trephine biopsy sections most often show an interstitial infiltrate [232]. 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 [199]. Reticulin is moderately increased, being less heavy than in HCL [199] so that marrow can usually be aspirated. CD72 is expressed but not annexin A1. Splenic infiltration is in the red pulp with a minority of cases showing blood lakes [232].

Cytogenetic and molecular genetic analysis

Complex karyotypes are common as is monoallelic deletion of *TP53* [199]. *BRAF* mutation is absent [225]. *MAP2K1* mutations have been described [230].

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.

Hairy cell leukaemia, Japanese variant

A Japanese variant of HCL has been described [233–235]. The clinical course is indolent and the condition may be responsive to cladribine therapy.

Clinical, haematological and cytological features

The Japanese variant is more common in women than in men. There is usually splenomegaly without peripheral lymphadenopathy. The lymphocyte count is usually increased. The neoplastic cells have plentiful, weakly basophilic hairy cytoplasm; they have homogeneous condensed chromatin with indistinct nucleoli. The bone marrow is usually easy to aspirate. TRAP activity is weak or negative. Ultrastructural examination shows the presence of ribosomal-lamellar complexes.

Immunophenotype

The immunophenotype is of a mature B cell but with weak or negative SmIg. Expression of CD22 and CD11c is usual. CD25 is negative and CD103 is usually negative.

Histology

Trephine biopsy sections usually show an interstitial infiltrate.

Splenic marginal zone lymphoma including splenic lymphoma with villous lymphocytes

Splenic lymphoma with villous lymphocytes (SLVL), first described in 1987 [236], 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 [237]. In the WHO classification SLVL is recognized as a presentation of SMZL [238]. 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 [199]. 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 [239] and transformation of SMZL in 13% [240]. 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 [236,240–242]. 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 [242]. 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. 7.24). The cytoplasm varies in amount, is moderately basophilic and has short villous projections, sometimes localized at one pole of the cell. Aggregation of lymphocytes in the peripheral blood film has been reported in a number of patients [243]. The nucleocytoplasmic ratio is higher than in HCL or hairy cell leukaemia variant. A minority of cells show plasmacytoid features, that is the nucleus is

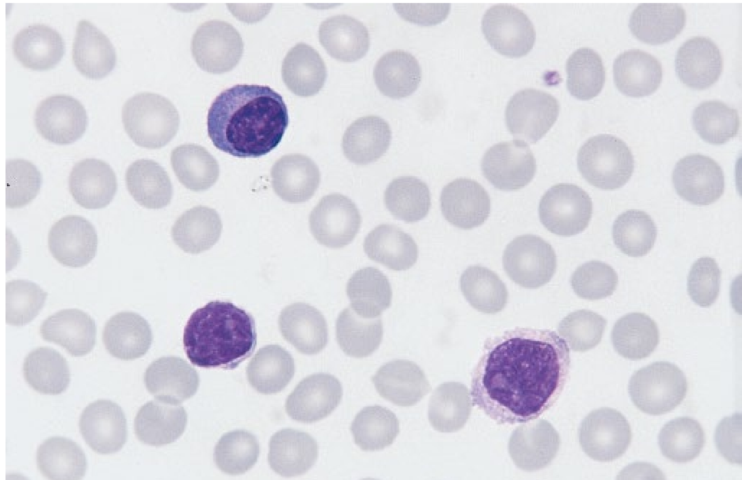


Fig. 7.24 PB film in splenic lymphoma with villous lymphocytes. The cells have small, inconspicuous nucleoli. One has villous cytoplasm and one is showing plasmacytoid differentiation. MGG ×100.

eccentric, basophilia is more 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 AIHA and 2% an autoimmune thrombocytopenia [239]. Other autoantibodies that have been reported include the lupus anticoagulant and anticardiolipin antibodies [240].

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 [239,240].

Poor prognostic factors for disease-specific survival are anaemia and a lymphocyte count of more than 16×10^9/l [239].

Immunophenotype

The SLVL/SMZL cell corresponds to a relatively mature B cell with some features suggesting plasmacytoid differentiation (see Table 7.8). SmIg is strongly expressed (IgM and usually IgD), and cytoplasmic immunoglobulin and CD38 [65] 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 [189]. 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 and CD103 (in one study 23% and 6%, respectively, were positive) [241,242]. CD5-positive cases have a significantly higher lymphocyte count [244]. Cases have been reported with CD5 positivity of circulating cells with cells in the spleen being CD5 negative [245]. CD43 is usually weak or negative [66]. CD11c is expressed in about 50% of patients [241,242], CD25 in about a quarter [56,241] and CD72 in the majority [242]. Expression of CD200 is usually weak [246]. 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 and interstitial or, in advanced disease, diffuse. The presence of multiple nodules is most characteristic [199]. Nodules in the bone marrow may have a central reactive germinal centre [240]. 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 [4,194,247]. 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 analysis

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 [240,248]. A t(11;14)(p11;q32) translocation has been observed in a small number of patients and has been associated with an adverse prognosis [249], and other translocations with a 14q32 breakpoint have occasionally been found. Rearrangement of the IGH locus and *TCL1A* associated with t(14;14)(q32.13;q32.33) or a variant is a recurring abnormality reported in a condition that appears likely to be SLVL [250]. Translocations with 7q22 and 2p11 breakpoints are observed whereas trisomy 12 and 13q14 abnormalities on conventional cytogenetic analysis are uncommon [251]. Del(13)(q14) is more often observed by FISH

[252]. Molecular mechanisms of oncogenesis in patients with 7q abnormalities include dysregulation of the *CDK6* gene by proximity to IGH in t(7;14)(q21.2;q32) and by proximity to κ in t(2;7)(p12;q21.2). Loss of 7q22-36 (particularly 7q31-32) is found in as many as 40% of patients [240]. Abnormalities of 3q have been found in 10–20% of patients [240]. 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 [253]. A less common recurring cytogenetic abnormality is i(17q) [253]. *RB1* deletion may be detected by FISH analysis [252]. A minority of cases (less than 20%) have *TP53* (17p13) deletion or mutation, which is associated with a worse prognosis [254]. Somatic hypermutation of *IGHV*, a feature of normal splenic marginal zone B cells, has been found in about 50% of patients [240]. Other genes that may be mutated include *NOTCH2* and *KLF2* [238].

Cytogenetic or molecular genetic features associated with a worse prognosis include loss or dysfunction of *TP53*, 7q22-36 loss and unmutated *IGHV* genes [240], and possibly *NOTCH2* and *KLF2* mutation [238].

Problems and pitfalls

Diagnosis can be difficult because of the lack of distinctive immunophenotypic and cytogenetic features. The cytoplasmic 'villi' can also be infrequent. The differential diagnosis includes the provisional WHO entity, splenic diffuse red pulp small B-cell lymphoma, which may also have villous lymphocytes [238]; the presence of intrasinusoidal lymphoma cells is not helpful since it can be present in both these conditions.

Follicular lymphoma

Follicular lymphoma is a chronic B-lineage lymphoproliferative disorder with a growth pattern in lymph nodes that 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 [255]. Grade 3b follicular lymphoma appears to be a somewhat different disease from grades 1–3a and usually has areas of diffuse large B-cell lymphoma [255]. An increased incidence of follicular lymphoma has been reported in association with pesticide or herbicide exposure [255]. There is an increased incidence in patients with a family history of NHL [255]. The natural history of follicular lymphoma includes transformation to high grade lymphoma. Rarely there is transformation to lymphoblastic leukaemia/lymphoma or classical Hodgkin lymphoma [255].

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. Sometimes there is involvement of Waldeyer's ring, the gastrointestinal tract, ocular adnexae and soft tissues including breast. Occasionally the disease is diagnosed following an incidental blood film when there are no abnormalities on physical examination.

Circulating lymphoma cells were present in 11% of a large series of patients ($n = 533$) [256]. The neoplastic cells are more pleomorphic than CLL cells. 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. 7.25). Rarely lymphoma cells contain crystalline inclusions [257]. Presentation in leukaemic phase is not necessarily adverse if there

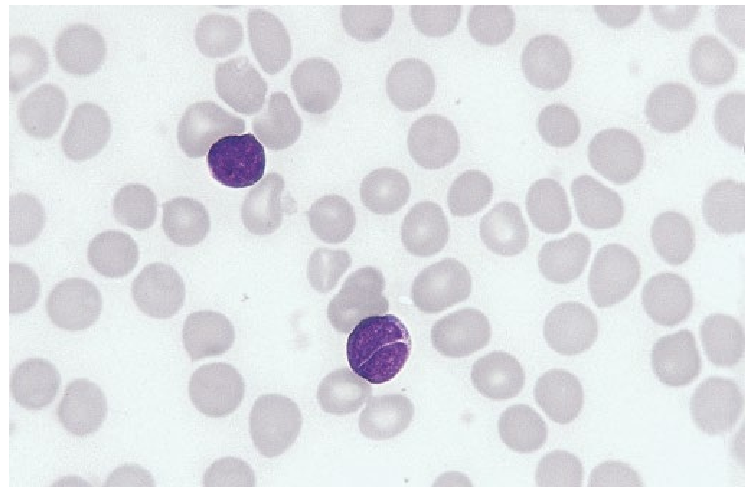


Fig. 7.25 PB film in the leukaemic phase of follicular lymphoma. One cell is very small with scanty cytoplasm; the other is nucleolated and has a deep, narrow cleft. MGG ×100.

is not a high disease burden [258] and with current treatment [256].

Follicular lymphoma may transform to a large cell lymphoma or, rarely, to Burkitt lymphoma or a lymphoblastic lymphoma/acute lymphoblastic leukaemia [259]. Burkitt transformation is related to the acquisition of a second chromosomal rearrangement, either t(8;14) or t(8;22), leading to *MYC* dysregulation [260].

In addition to the prognostic factors included in the Follicular Lymphoma International Prognostic Index (FLIPI) (see Table 7.6), multivariate analysis shows bone marrow infiltration, male gender and a lymphocyte count of less than 1.0×10^9/l to be associated with a worse prognosis [13]. An absolute monocyte count at presentation of 0.57×10^9/l or more [261] or greater than 0.63×10^9/l [262] is associated with a worse prognosis. The presence of circulating lymphoma cells has been associated with a worse prognosis [263], but see above.

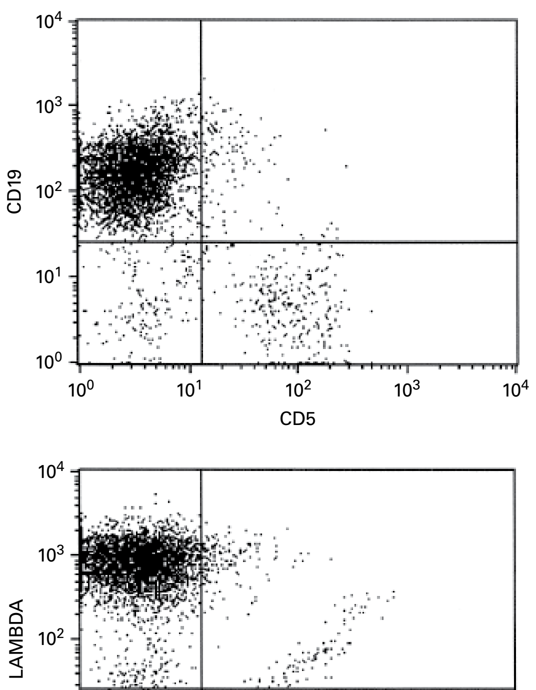


Fig. 7.26 Flow cytometric immunophenotyping in follicular lymphoma showing expression of CD19, strong expression of the λ light chain and lack of expression of CD5. (With thanks to Mr Ricardo Morilla, London.)

Immunophenotype

The characteristic immunophenotype (Figs 7.26 and 7.27) is positivity for B-cell-associated antigens such as CD19, CD20, CD22 and CD24 and positivity for FMC7. The expression of CD19 is often weaker than on normal B cells [190]. CD79b is usually positive (around 80% of cases) [189] and CD10 is often positive. The detection of CD10 positivity appears to be dependent on the specific antibody-fluorochrome used, in one study being

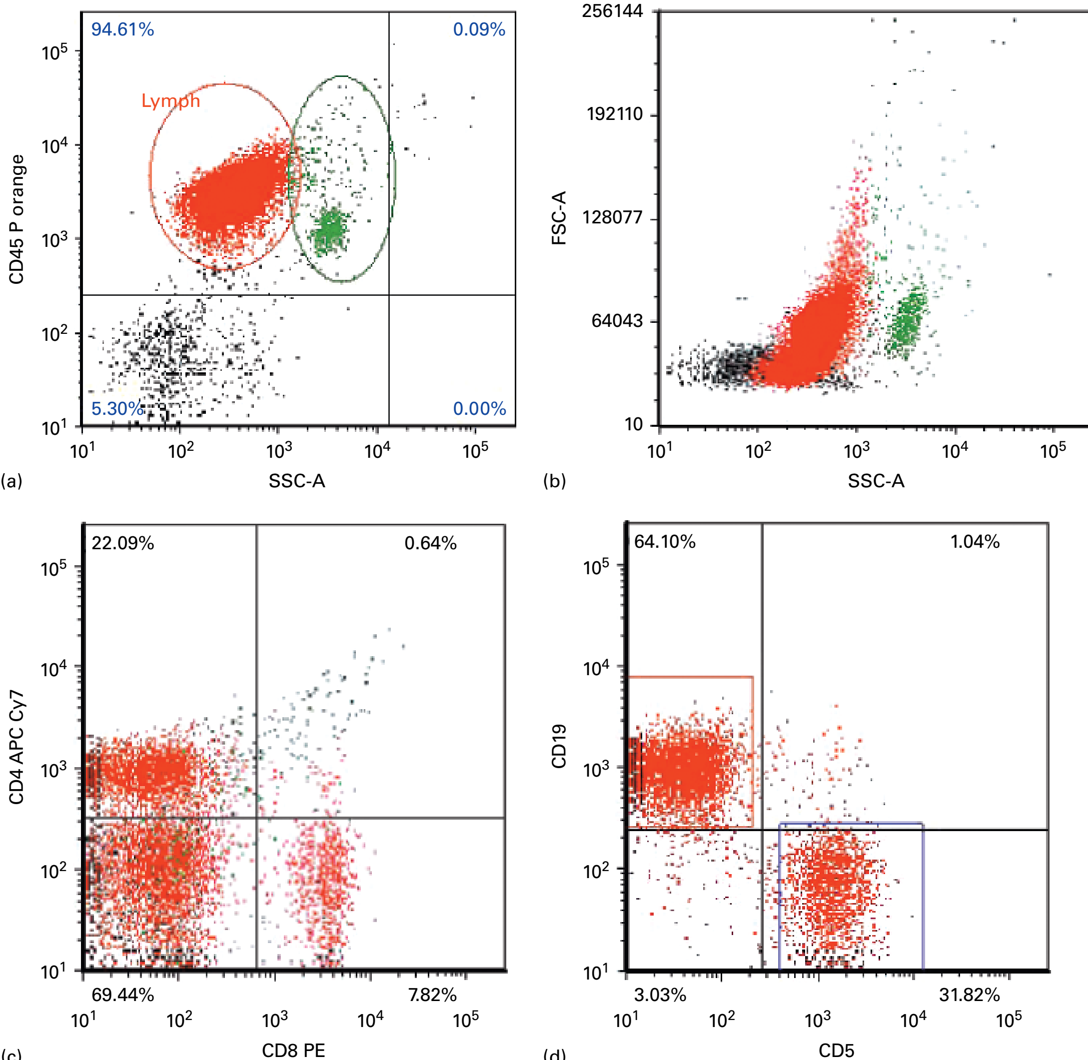


Fig. 7.27 Scatter plots showing flow cytometric immunophenotyping in follicular lymphoma: (a) CD45 against side light scatter (SSC) with gating on clusters corresponding to lymphocytes (red) and granulocytes (green); (b) forward light scatter (FSC) against SSC with gating on clusters corresponding to 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; (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 expresses CD10; (h) CD10 against λ confirming that the abnormal λ-expressing clonal B cells express CD10. APC, allophycocyanin; FITC fluorescein isothiocyanate; PE, phycoerythrin. (With thanks to Dr Helen Wordsworth and Sullivan Nicolaides Pathology, Brisbane.)

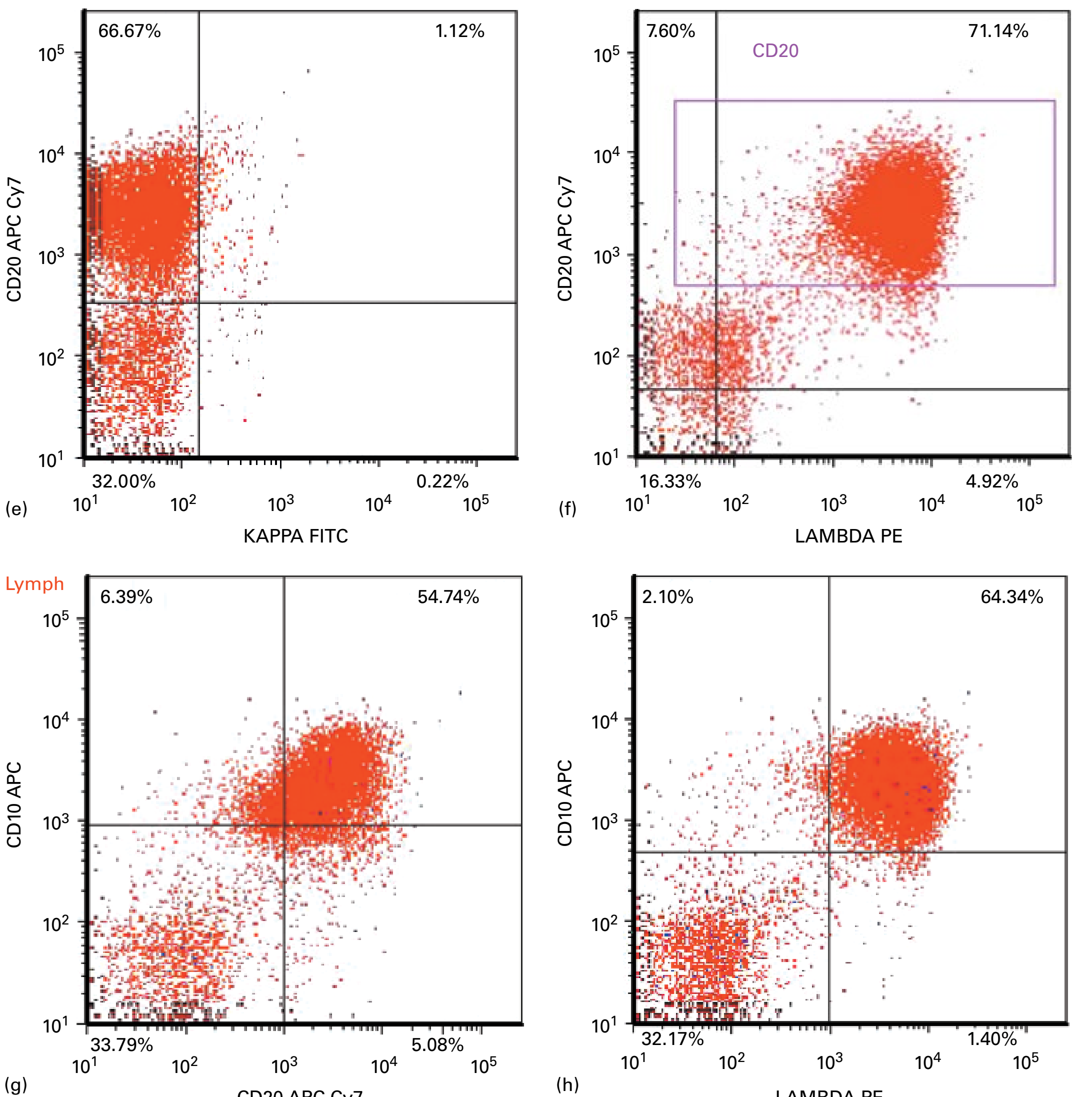


Fig. 7.27 (Continued)

observed to be 100% with one reagent and 0% with another [264]. Use of phycoerythrin (PE) rather than fluorescein isothiocyanate (FITC) is advised, to increase the probability of detecting a weaker reaction [265]. CD10 expression is less often detected in the bone marrow and peripheral blood than in the lymph node [256]. CD5 is not expressed. CD23, CD43 and CD11c are usually negative, although CD43 may be positive in some higher grade follicular lymphomas [66]. On flow cytometry, BCL2 expression is strong whereas reactive B cells show weak expression [266]. SmIg is strongly expressed. IgM is most often expressed but IgG and IgA expression are also quite common; IgD is not expressed [267]; κ expression is more common than λ expression.

Histology

Lymph node histology shows a follicular growth pattern. BCL2 is expressed in the follicles;

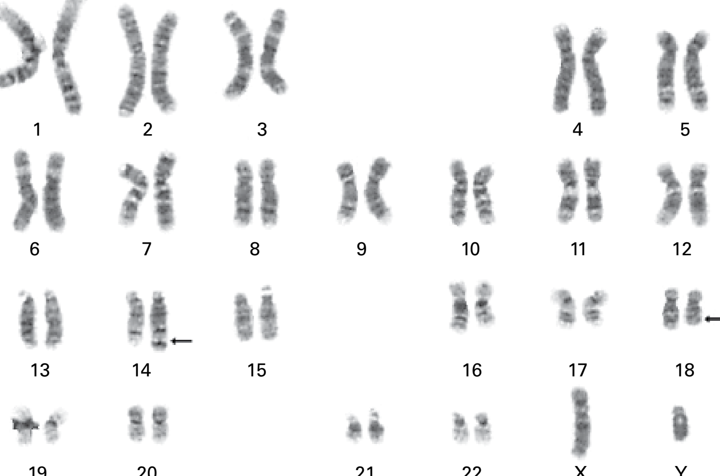


Fig. 7.28 A karyogram showing t(14;18)(q32;q21.3). (With thanks to Dr Fiona Ross, Salisbury.)

expression is less common in those with grade 3 histology. However, cases with *BCL6* rather than *BCL2* rearrangement also lack *BCL2* expression [268]. *BCL6* is expressed, whether or not the gene is rearranged [255]. MUM1/IRF4 is usually negative [255].

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 [266]. A bone marrow aspirate may be normal in the presence of an abnormal trephine biopsy. Spleen involvement is in the white pulp.

Cytogenetic and molecular genetic analysis

Follicular lymphoma arises from a post-germinal centre B cell showing somatic hypermutation. The most characteristic cytogenetic abnormality is t(14;18)(q32;q21.3) (Fig. 7.28). Proximity to the IGH locus or associated mutation dysregulates the *BCL2* oncogene at 18q21.3 rendering the cells resistant to apoptosis. In the variant translocations, t(2;18)(p11.2;q21.3) and t(18;22)(q21.3;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 [268]. There are two clusters of breakpoints in the *BCL2* gene, designated 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 with MBR breakpoints and worst when neither breakpoint is detected [269]. *BCL2* rearrangement can be detected by both PCR and RT-PCR [270]. 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;q21.3) and variant translocations can be detected by single-colour FISH, with a probe that encompasses the breakpoint on chromosome 18. The t(14;18)(q32;q21.3) translocation 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) [271], der(1)t(1;17)(p36;q11-21) and der(1)t(1;11)(p36;q13) [272]. Rearrangement of *BCL6*, most often resulting from t(3;14)(q27.3;q32), appears to be an alternative oncogenic mechanism in cases lacking rearrangement of *BCL2* [268]. In addition,

dual *BCL2* and *BCL6* translocations are seen in around 10% of patients; the translocation involving *BCL6* can sometimes be shown to be a second event [273]. 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 [274]. Trisomy 7 has been related to the large cell histology and rearrangements with an 8q24 breakpoint to a blastoid variant [271]. Rearrangement of a gene now designated *BCL5* at 17q22 correlates with transformation to more aggressive disease [275]. Disease progression can also be associated with acquisition of t(1;22)(q23.3;q11) in which the *FCGR2B* (*FcγRIIB*) gene is brought under the influence of positive regulatory elements of the λ gene [276]. There may be mutation or copy number alteration of *TNFRSF14*, *EZH2*, *CREBBP*, *RRAGC*, *KMT2D* and a number of other genes may be mutated [255].

Molecular genetic abnormalities that may appear with disease progression include: loss of 6q25-26 [274]; loss of *TP53*, which correlates with del(17p); loss of *CDKN2A* and *CDKN2B*, which correlates with del(9)(p21); and activation of *MYC*. Follicular lymphoma is quite uncommon in children but when it occurs the proportion of patients with *BCL2* rearrangement and *BCL2* expression is lower than in adults [277]; 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. There is an increased incidence associated with a family history of mantle cell lymphoma or other NHL [278].

Clinical, haematological and cytological features

Mantle cell lymphoma is principally a disease of middle and old age (median age 60 years) with a marked male predominance [279]. Presentation is usually with advanced stage disease, most often with lymphadenopathy and splenomegaly and often with extranodal disease, including involvement of Waldeyer's ring, lung, pleura and the gastrointestinal tract – multiple lymphomatous polyposis [278,280]. There is peripheral blood involvement in about two-thirds of patients and this has been considered indicative of a worse prognosis [281]. However, leukaemic non-nodal mantle cell lymphoma, which usually involves the spleen, is usually clinically indolent [282]. 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 [283].

Cells are usually pleomorphic and most often mainly medium sized with irregular nuclei [284] (Figs 7.29 and 7.30. Some show inconspicuous nucleoli or pronounced nuclear indentations or clefts. Cytoplasmic vacuoles may be present [285]. Rarely there are cytoplasmic granules [286]. There may be some cells with a diffuse chromatin pattern, and in occasional patients these cells predominate [280]. 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, thus resembling ALL or there may be some more mature cells (Figs 7.31 and 7.32). The blastoid variant usually comprises

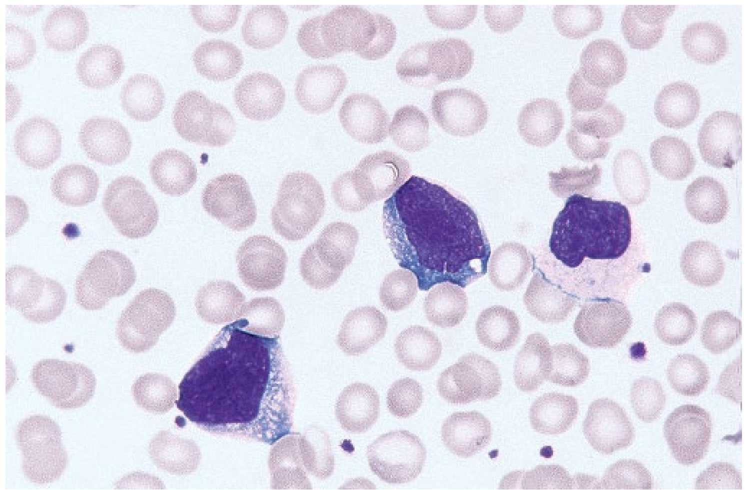


Fig. 7.29 PB film in mantle cell lymphoma. The cells are markedly pleomorphic. MGG ×100.

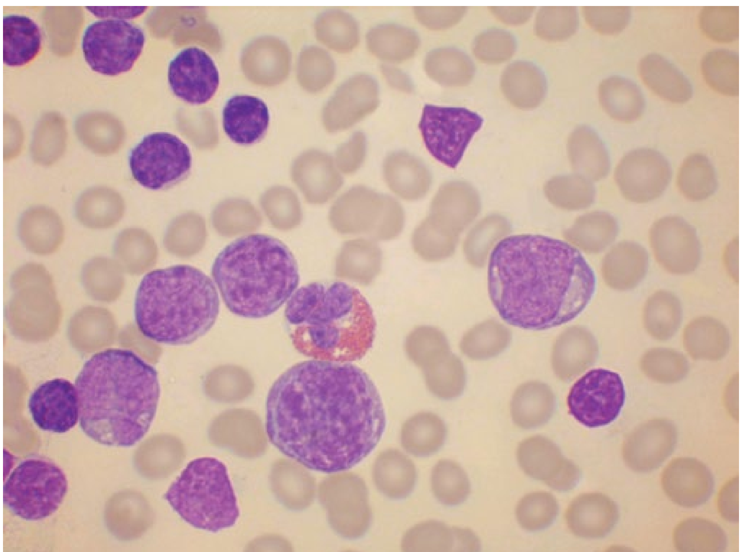


Fig. 7.30 PB film from a patient with mantle cell lymphoma showing a range of cells from small lymphocytes resembling those of CLL to larger cells with irregular nuclei and ill-defined nucleoli; there are some smear cells. MGG ×100.

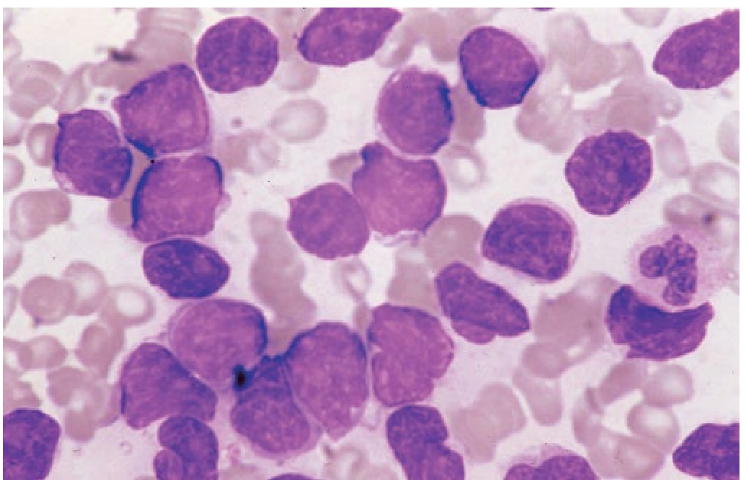


Fig. 7.31 PB film in blastoid variant of mantle cell lymphoma showing medium-sized cells with a high nucleocytoplasmic ratio; some have a diffuse chromatin pattern and others show some degree of chromatin condensation. Some cells have distinct small or medium-sized nucleoli. MGG ×100.

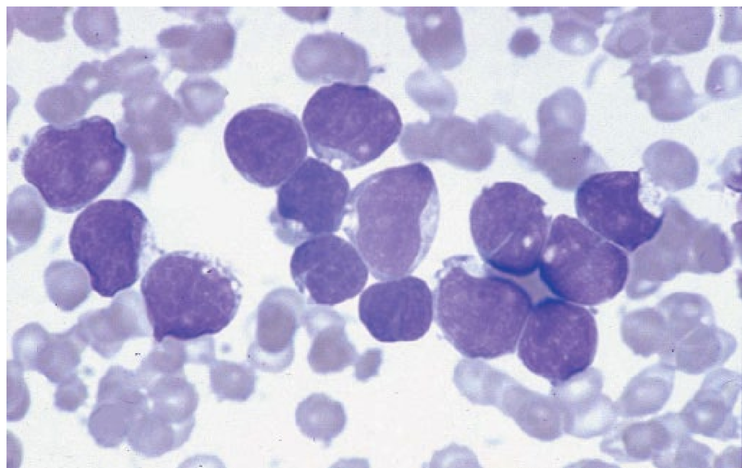


Fig. 7.32 PB film from a patient with blastoid variant of mantle cell lymphoma showing pleomorphic cells ranging from mature lymphocytes to blast-like cells. Some cells have cleft nuclei and some have cytoplasmic vacuoles. MGG ×100.

10–20% of cases, though in some series it has been as high as 40% [279,283,287]. In some patients, lymphoma cells have the cytological features of prolymphocytes [196]. Some patients, around 15% of cases, have mainly small cells with only a minority of cells having typical mantle cell features [283]. Platelet satellitism of lymphoma cells has been described [288]. Following splenectomy, the WBC often falls [40]. Lymphoma cells are mobilized into the peripheral blood by ibrutinib therapy, particularly in patients with bone marrow involvement and with the degree of lymphocytosis correlating with the extent of bone marrow infiltration [289]. Patients presenting with typical mantle cell lymphoma features may have a blastoid transformation with disease progression [283]. Rarely transformation is to Burkitt lymphoma [290,291].

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 CD10, CD11c, CD103 and BCL6 (see Fig. 2.4). Expression of CD20 is strong, being stronger than expression of CD19. There is failure to express CD5 in 10–15% of patients. CD23 is expressed in a quarter to a half of patients [292–294] but expression is weak [292,293] and it may therefore be more readily detected by flow cytometry than by immunohistochemistry. There may be discordant CD23 expression in different tissues [294]. CD23 expression correlates with a better prognosis [294]. CD25 is usually not expressed. CD38 is expressed on more than 20% of cells in the majority of patients [295], and correlates with a worse prognosis [296]. CD43 has been variously stated to be weak or negative [295], usually positive [278] or usually strong [66,297]. CD45 expression is strong. CD200 is usually negative [278]. There may be aberrant expression of myeloid and T-lineage antigens such as CD2, CD7, CD13 and CD33 [298]. SmIg is moderately strongly expressed. It is usually IgM with or without IgD. λ is more commonly expressed than κ [267]. 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 [299,300]. However, the detection of nuclear cyclin D1 by flow cytometry may be neither very sensitive (in one study positive in only two-thirds of patients) nor totally specific (in the same study also positive in a fifth of patients with CLL) [300].

Histology

Bone marrow infiltration is common, being observed in up to 80% of patients [281]. 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 [279]. 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 [301], being detectable in almost all cases [302]. Nuclear expression of SOX11 is also diagnostically useful since it is demonstrable also in cases that lack rearrangement of *CCND1* and thus do not express cyclin D1 [201]. A higher proliferative fraction (Ki-67 expression) and expression of TP53 are indicative of a worse prognosis [302]; both are more often observed in the blastoid variant [287].

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 analysis

The characteristic cytogenetic abnormality is t(11;14)(q13.3;q32) (Fig. 7.33) 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 [303]. 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 [304]. Alternatively, specific

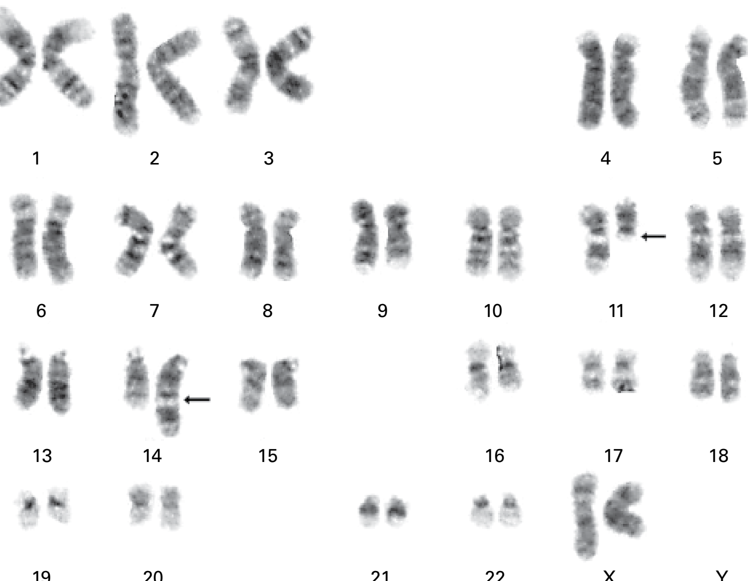


Fig. 7.33 A karyogram showing t(11;14)(q13.3;q32). (With thanks to Dr Fiona Ross.)

probes for *CCND1* and the IGH locus 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. RT-PCR is also applicable [270] but false-negative results occur. A minority of cases of mantle cell lymphoma show a variant translocation, t(11;22)(q13.3;q11.2), 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 possibly cyclin D3. For example, patients have been described in whom cyclin D2 overexpression resulted from t(2;12)(p11.2;p13.3), bringing *CCND2* (encoding cyclin D2) under the influence of the κ locus [305], or t(12;14)(p13.3;q32), bringing *CCND2* under the influence of the IGH locus [306].

It is unusual for t(11;14) to be present as the sole abnormality [307]. In one study 85% of patients had additional chromosomal abnormalities [287]. 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 [302,307–311]. 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 [287,312] but not in a third series [307]. Burkitt lymphoma-related translocations, both t(8;14)(q24.2;q32) and t(2;8)(p12;q24.2), have occurred in blastoid transformation [313] and in transformation to Burkitt lymphoma [290]. 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 [307]. Trisomy 12 [314], add(Xq) [315], del(17p) [315] or specifically del(17)(p13) [287], del(9)(p21) [287], add(3q) [279] and complex karyotypes [314] have been associated with a worse prognosis.

Cells of mantle cell lymphoma usually show unmutated *IGHV* (pre-germinal centre origin) genes, but in a significant minority there is *IGHV* somatic hypermutation [278]. 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 [316]. In addition to dysregulation of the *CCND1* gene, there is often amplification of 3q28q29 suggesting that another oncogene at that site may be relevant [315]. Amplification of part of 6p is also common [315]. 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) [315,317]. Del(8p) and del(13)(q34) correlate with a worse prognosis [317].

Secondary genetic abnormalities are common, gains of *MYC*, *CDK2*, *CDKN1B* and *MDM2* being seen in 10% of 42 patients in one study, and loss of *RB1*, *CDKN2A*, *ATM* or *TP53* in 38%, 31%, 24% and 10% respectively [318]. *CDKN2A* mutation and *TP53* deletion are prognostically adverse. *ATM* function may be lost by point mutation or deletion [278,309]; in patients with loss of one allele as a result of del(11)(q23) the other allele is often mutated [138]. 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 [310] but, in a second study, association with leukaemic manifestations was not observed [317]. Patients with the blastoid variant often have mutation of *TP53*, *CDKN2A* (encoding $p16^{INK4A}$) and *CDKN2C* (encoding $p18^{INK4C}$) [315]. Mutation of *CDKN1A* (encoding p21) is also associated with worse prognosis [319]. Expression of TP53 correlates with *TP53* mutation and is indicative of a worse prognosis [296].

Prognosis

Many adverse prognostic indicators are known [279]. These include more advanced age, worse performance status, B symptoms, high WBC, peripheral blood involvement (except in patients without lymphadenopathy), higher lactate dehydrogenase (LDH), blastoid and pleomorphic cytological variants, higher proliferation fraction or mitotic index, and the adverse karyotypic and molecular abnormalities described above. An absolute monocyte count of less than 0.5×10^9/l has been associated with better survival in two series of patients [320].

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, this not infrequently represents CLL, 9/28 cases (32%) in one series [321], so that confirmation by other techniques, such as FISH or cyclin D1 or SOX11 expression, is indicated [201,295,322].

Burkitt lymphoma

'Acute leukaemia with Burkitt lymphoma cells' was first described in 1972 [323], 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 [324]. Human herpes viruses 5 and 8 and other oncogenic influences may also have a role in endemic Burkitt lymphoma [325].

Individuals with X-linked immunoproliferative disease are at high risk of Burkitt lymphoma.

In addition to *de novo* cases, Burkitt lymphoma can also occur as a transformation of a lower grade leukaemia or lymphoma, for example 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 or involvement of breast or ovaries) 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. Tumour lysis syndrome can occur on initiation of treatment or even without treatment.

Immunophenotype

Usually the lymphoma cells are mature B cells, expressing IgM, B-cell-associated antigens (e.g.

CD19, CD20, CD22, CD79a, CD79b and PAX5) plus CD10 and BCL6. CD34 and TdT are not expressed. CD38 is often strongly expressed. CD200 expression is weak [246]. Some cases, however, have the immunophenotype of ALL (common or pre-B) with expression of TdT and sometimes CD34 [326]. As their prognosis is very poor if they are classified and treated as ALL, it seems preferable that such cases be regarded as Burkitt lymphoma.

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, which is characteristic of Burkitt lymphoma but not pathognomonic. B-cell markers including PAX5 are expressed. There is often expression of CD38, CD43 and CD77. Immunohistochemical demonstration of expression of BCL6 but not BCL2 supports the diagnosis of Burkitt lymphoma; BCL2 is weakly positive in only 20% of patients [325]. MYC is usually expressed [325]. Demonstration of expression of the proliferation marker, Ki-67, is important in confirming the diagnosis; expression approaches 100%.

Cytogenetic and molecular genetic analysis

The majority of patients have t(8;14)(q24.2;q32) with juxtaposition of *MYC* to the IGH locus (see Fig. 2.13). In a minority of patients there is t(2;8)(p11.2;q24.2) or t(8;22)(q24.2;q11.2) with juxtaposition of *MYC* to the κ and λ loci respectively. These rearrangements can be demonstrated by FISH (Fig. 7.34). The gene expression profile is distinctive.

Prognosis

Adverse prognostic features include bone marrow and central nervous system disease, a high LDH and a large unresected tumour mass [325].

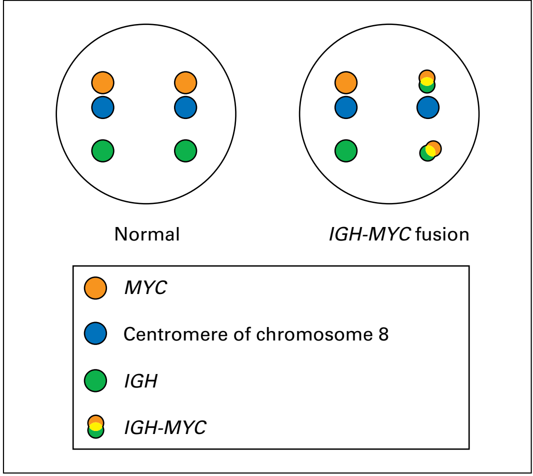


Fig. 7.34 Diagrammatic representation of tricolour, dual-fusion FISH for the detection of IGH-*MYC* juxtaposition, using an orange *MYC* probe, a green IGH probe and a blue probe for the centromere of chromosome 8. The normal cell has two orange *MYC* signals, two blue centromeric signals and two green IGH signals. The cell with IGH/*MYC* juxtaposition as a result of t(8;14)(q24.2;q32) 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)(p11.2;q24) as well as with the more common t(8;14)(q24.2;q32).

Problems and pitfalls

L3 cytological features do not always equate with Burkitt lymphoma. Except in the case of endemic Burkitt lymphoma, immunophenotyping and genetic analysis are usually essential to confirm the diagnosis. Burkitt lymphoma needs to be distinguished from a high grade lymphoma in which a translocation involving *MYC* coexists with a *BCL2* or *BCL6* rearrangement, or both (which often has bone marrow involvement). Such cases may have morphological features very similar to Burkitt lymphoma or intermediate between Burkitt lymphoma and diffuse large B-cell lymphoma.

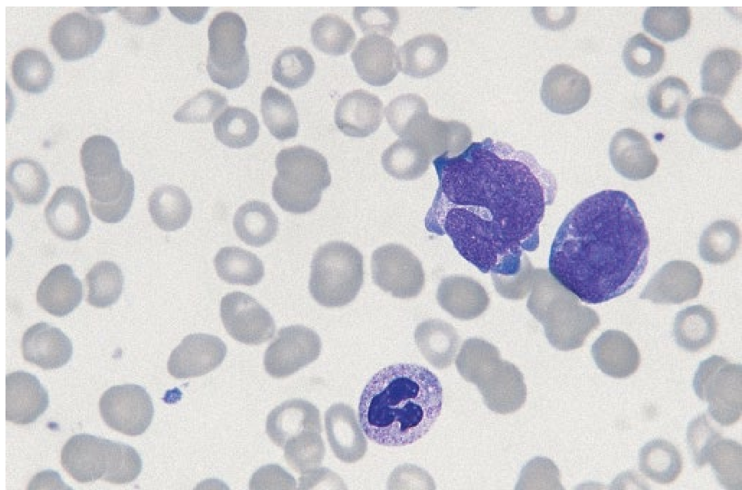


Fig. 7.35 PB film in diffuse large B-cell lymphoma of centroblastic type. MGG ×100.

Conversely, the immunophenotype is not infrequently atypical (e.g. lack of expression of SmIg or lack of expression of one or more B-cell antigens – CD19, CD20, CD22 or CD79a) or CD10, found in 4 of 35 cases [327], and observation of L3 morphology should therefore lead to genetic analysis even when the immunophenotype is atypical.

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 2016 revision of the WHO classification recognizes DLBCL, not otherwise specified [328] 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 [329]. 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. Cytologically DLBCL cannot be distinguished from T-cell lymphoma. 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. 7.35). 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 [330] (Figs 7.36 and 7.37). Aggregates of lymphoma cells in a peripheral blood film have also been reported in primary cutaneous leg-type B-cell lymphoma [331].

Immunophenotype

Flow cytometry shows high forward angle light scatter [209]. 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

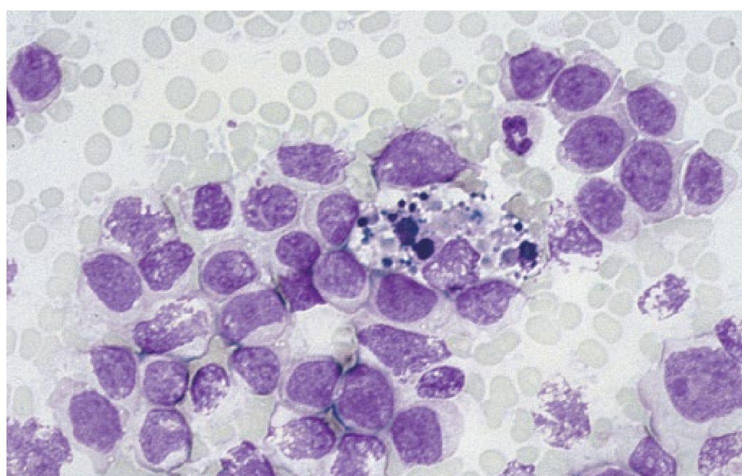


Fig. 7.36 A clump of lymphoma cells and a macrophage in a blood film of a patient with intravascular large B-cell lymphoma. The blood film was prepared from the tip of the needle following phlebotomy. MGG ×100. (With thanks to Dr Ralph Cobcroft, Brisbane.)

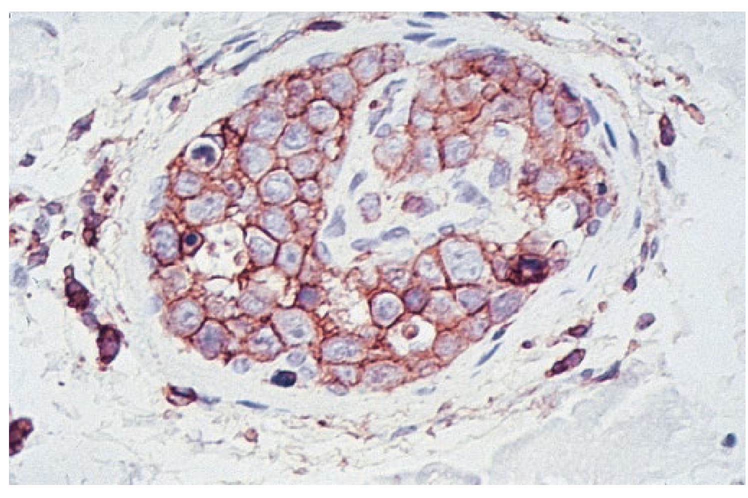


Fig. 7.37 A skin biopsy from a patient with intravascular large B-cell lymphoma showing cohesive masses of lymphoma cells within a capillary (same patient as Fig. 7.36). The cells were CD45 and CD20 positive and S100 negative. Immunohistochemistry with an anti-CD20 monoclonal antibody. (With thanks to Dr Ralph Cobcroft and with permission of the *British Journal of Haematology*.)

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 [264]. CD71 is usually expressed [209]. There may be a failure to express SmIg [208]. CD200 expression varies from weak to strong [246], being positive in about a fifth of cases, particularly those of activated B-cell-like type [59].

Histology

The bone marrow is consistently involved in patients with circulating lymphoma cells but the pattern of infiltration varies; there may be discordant involvement by low grade lymphoma. Lymph nodes usually show diffuse infiltration but occasional cases have a focal follicular pattern [328]. There is expression of CD20 and CD79a and, in some patients, CD5, CD10, CD30, BCL2, BCL6, IRF4/MUM1 or TP53 [328].

Cytogenetic and molecular genetic analysis

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 3q27.3, including t(3;14)(q27.3;q32). Other cases show miscellaneous cytogenetic abnormalities including some with 14q32 (IGH) or 8q24.2

(*MYC*) involvement. Dual-colour FISH with probes for *BCL6* and IGH can be used to detect t(3;14)(q27.3;q32) [332]. 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 (cyclophosphamide, doxorubicin, vincristine, prednisolone) type with or without rituximab.

Prognosis

Adverse prognosis is associated with higher patient age, poor performance status, more advanced stage or more bulky disease, higher LDH, bone marrow involvement and an activated B-cell-like rather than germinal centre gene expression profile or equivalent immunophenotypic profile. A reduced lymphocyte count ($<0.8 \times 10^9$/l) has also been found to be prognostically adverse [333].

Problems and pitfalls

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.

Lymphoplasmacytic lymphoma

Lymphoplasmacytic 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. 7.38). As defined in the WHO classification, this lymphoma shows some differentiation to mature plasma cells. Hepatitis C infection and autoimmune disorders predispose. There may be a familial predisposition.

Waldenström macroglobulinaemia is now regarded as a subset of lymphoplasmacytoid lymphoma with bone marrow infiltration and an IgM paraprotein [334]; in Waldenström's

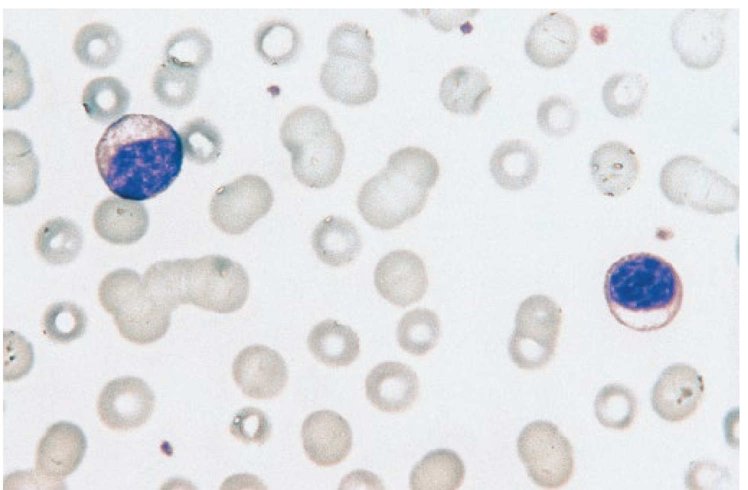


Fig. 7.38 PB film in Waldenström macroglobulinaemia; this term describes a lymphoplasmacytoid lymphoma with production of monoclonal IgM. The blood film shows two plasmacytoid lymphocytes together with rouleaux and abnormal staining characteristics consequent on the high level of IgM. MGG ×100.

initial description of the condition the paraprotein was present in a high concentration. Cold haemagglutinin disease is a distinct entity [334,335].

Clinical, haematological and cytological features

Clinical features can include splenomegaly, lymphadenopathy and sometimes features of hyperviscosity or cryoglobulinaemia. The peripheral blood may show increased lymphocytes, plasmacytoid lymphocytes or plasma cells. 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. Occasionally lymphoma cells contain cytoplasmic crystals, Dutcher bodies or Russell bodies. The bone marrow may show increased mast cells, in addition to a lymphoid infiltrate, which may be interstitial, nodular, paratrabecular or diffuse.

Immunophenotype

The immunophenotype is that of a late B cell or plasma cell with expression of cytoplasmic immunoglobulin in at least some cells. SmIg is expressed more strongly than in CLL cells. CD11c, CD19, CD20, CD22 and CD79a are expressed, with CD20 being more strongly expressed than in CLL [336]. CD5, CD10 and CD23 are not usually expressed (about 5% of cases are CD5 positive). FMC7 was positive in 38% of 29 patients in one study [337]. CD38 may be expressed but expression is weaker than in plasma cells [65]. 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 [265].

Cytogenetic and molecular genetic analysis

The t(9;14)(p13.2;q32) translocation in which the transcription factor gene, *PAX5*, is dysregulated by proximity to IGH was reported as characteristic [338] but has been found to be actually quite uncommon [339]. Trisomy 4 is found in a fifth of patients [334]. The most characteristic feature is mutation of *MYD88*, present in 90% of cases. Associated *CXCR4* mutation is common and, less often, *ARID1A* mutation is present [334].

Problems and pitfalls

It should be noted that plasmacytic differentiation can occur in other types of NHL, for example in SMZL. Monoclonal gammopathy of undetermined significance with an IgM paraprotein should be distinguished; it has no clinical features, an IgM paraprotein of less than 30 g/l and less than 10% lymphoplasmacytoid cells in the bone marrow.

Heavy chain diseases

The heavy chain diseases are rare lymphoproliferative disorders characterized by synthesis of a defective immunoglobulin heavy chain. In the case of γ and μ heavy chain diseases, the peripheral blood and bone marrow may be involved [340,341].

Haematologically, μ heavy chain disease resembles CLL but clinically there is usually hepatosplenomegaly without peripheral lymphadenopathy [341]. The bone marrow aspirate shows not only small lymphocytes but also vacuolated plasma cells [340]. Bence–Jones proteinuria is present in around half of patients [340].

Clinically and histologically, γ heavy chain disease resembles lymphoplasmacytic lymphoma [341]. Association with a wide range of autoimmune diseases is common. The peripheral blood features may resemble CLL or there may be plasmacytoid lymphocytes or plasma cells. The bone marrow may show increased plasma cells or plasmacytoid lymphocytes.

Other B-cell non-Hodgkin lymphomas in leukaemic phase

Various other B-cell lymphomas may have a leukaemic phase but this is quite uncommon. This has occasionally been reported in marginal zone lymphoma including nodal marginal zone

lymphoma (monocytoid B-cell lymphoma) [342] and MALT (mucosa-associated lymphoid tissue) lymphoma [343]. Cytological features are variable and not distinctive. The immunophenotype is usually CD5 negative (only about 5% are positive) and CD10 negative [265]. There is often expression of CD11c (weaker than in HCL) and sometimes of CD103 [265]. There may be some plasmacytoid differentiation leading to expression of CD38 and CD138 in some cells [265].

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 [267], this disease being the tissue equivalent of CLL and classified with it.

Plasma cell leukaemia

Plasma cell leukaemia can occur *de novo* or as the terminal phase of multiple myeloma [344]. 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, support the view that *de novo* plasma cell leukaemia is a different disease from multiple myeloma [345]. Prognosis is poor in both *de novo* cases and secondary cases. In one series of 18 patients with *de novo* disease the median survival was only 7 months [346]. 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 [347].

The 2016 WHO criteria for a diagnosis of plasma cell leukaemia are that neoplastic plasma cells are 20% or more of circulating cells or that their absolute count is greater than 2×10^9/l [344].

Clinical, haematological and cytological features

There may be hepatomegaly, splenomegaly, lymphadenopathy, hypercalcaemia and renal insufficiency. In *de novo* cases lytic bone lesions and bone pain are less frequent than in multiple myeloma, and patients tend to be younger.

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. 7.39). 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. 7.40). In the latter

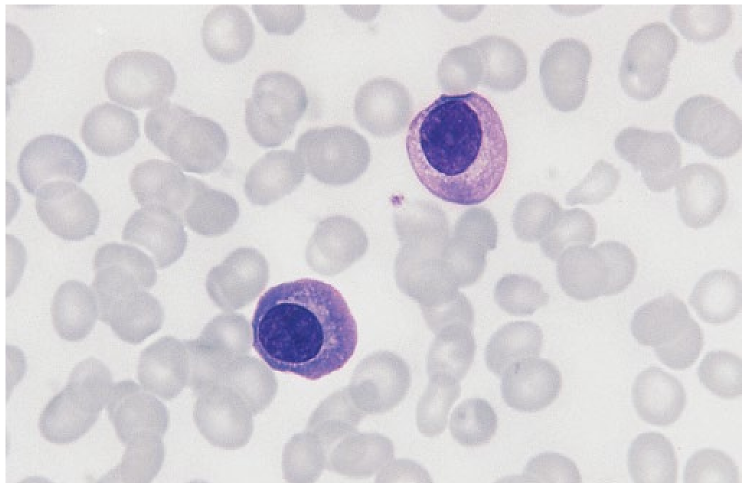


Fig. 7.39 PB film in plasma cell leukaemia. The malignant cells are identified as plasma cells by their eccentric nuclei and pale paranuclear area that represents the Golgi zone. MGG ×100.

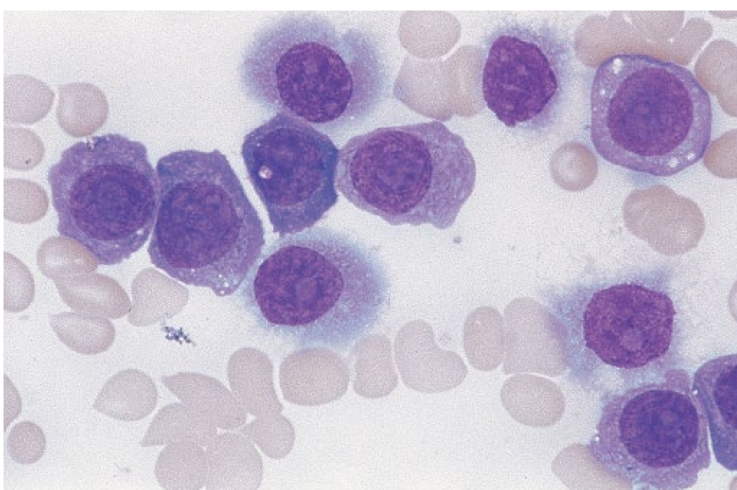


Fig. 7.40 PB film in plasma cell leukaemia with cells showing plasmablastic morphology. MGG ×100.

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 7.8, positive reactions are found with McAb that show some selectivity for plasma cells such as PCA-1, BU11 [4], CD38 and CD138 [348]. 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. HLA-DR may be expressed. There may be aberrant expression of CD13 [345] and CD28 (usually expressed on T cells) [265], and a failure to express CD27 [265].

The immunophenotype in *de novo* plasma cell leukaemia has been found to differ somewhat from that of multiple myeloma [345]. CD20 is more often expressed whereas CD9, CD56 and HLA-DR are less often expressed [344,345,347]. 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 [265,345,347].

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 analysis

Plasma cell leukaemia may show cytogenetic abnormalities similar to those of multiple myeloma including t(11;14)(q13.3;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 [345]. Hyperdiploidy is common in multiple myeloma but not in plasma cell leukaemia [345]. 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 [345].

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 7.16 and 7.17). 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. Lack of expression of CD56 or coexpression of CD16 and CD57 suggests that NK cells are neoplastic [349]. 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 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 [350], and CD158 (killer inhibitory receptor, KIR) antibodies can also be useful but

Table 7.16 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; positive in all except the earliest of T-lineage cells and in 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 also Table 7.7)
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 7.7
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

Table 7.17 Characteristic immunophenotype of chronic T-cell leukaemias.

Marker	LGLL – T cell	LGLL– NK	T-PLL	ATLL	Sézary syndrome
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; LGLL, large granular lymphocyte leukaemia; NK, natural killer cell; T-PLL, T-cell prolymphocytic leukaemia.

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 more than 20 entities recognized in the 2016 revision of the 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.

In a unique case, T-cell large granular lymphocyte leukaemia occurred in a child as a complication of early onset lymphoproliferation and autoimmunity associated with a germline gain-of-function mutation in *STAT3* [351].

Clinical, haematological and cytological features

T-cell large granular lymphocyte leukaemia occurs predominantly in the elderly [352,353]. About a third of patients are asymptomatic at the time of diagnosis [354,355]. Symptomatic patients usually present either with recurrent infection, resulting from neutropenia, with signs and symptoms of anaemia, or with fever, sweats and weight loss [356]. There is a strong association with Felty syndrome (rheumatoid arthritis with neutropenia and splenomegaly) and a less strong association with other autoimmune diseases [352] such as systemic lupus erythematosus, pure red cell aplasia and amegakaryocytic thrombocytopenia. Cyclical thrombocytopenia, attributable to cyclical disappearance of megakaryocytes from the bone marrow, has also been described [357]. Lymphadenopathy is

uncommon, but hepatomegaly and splenomegaly are frequent findings. Skin lesions are present in less than 20% of patients [358]. The disease typically has a prolonged survival with an actuarial median survival, in one series, of 166 months [355]. 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 [359]; this variant has been referred to as NK-cell-like T-cell lymphoma [360]. Rarely, spontaneous remission occurs [361]. Pregnancy may be associated with both a reduction in the lymphocytosis and an improvement in associated neutropenia [362].

Most patients have an increased WBC, lymphocytosis and an increase in large granular lymphocytes (LGL) [354,363]. 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 [364]. Lymphocytosis may appear only after splenectomy or with disease progression. The neoplastic cells are usually morphologically very similar to normal LGL (Fig. 7.41). 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. Rarely large, round or rod-shaped purple inclusions representing parallel tubular arrays have been described [365]. In some cases the majority of lymphocytes lack granules. In NK-cell-like T-cell lymphoma the cells are larger and more pleomorphic (Fig. 7.42). 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 [352]. 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 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

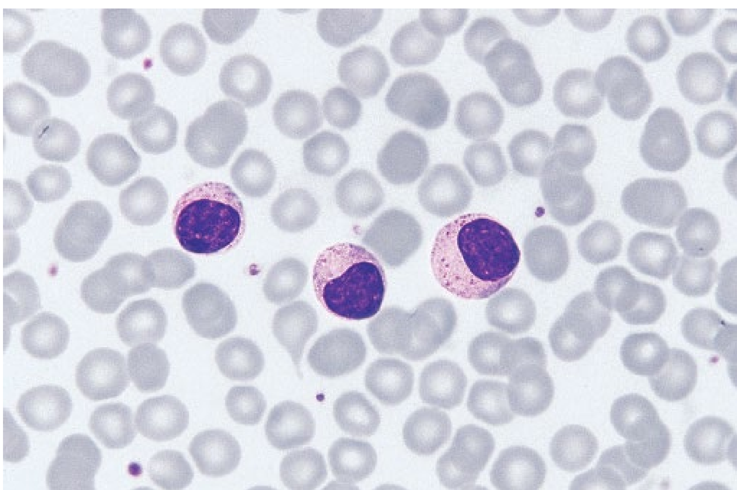


Fig. 7.41 PB film in large granular lymphocyte leukaemia. The cells have abundant weakly basophilic cytoplasm containing prominent azurophilic granules. MGG ×100.

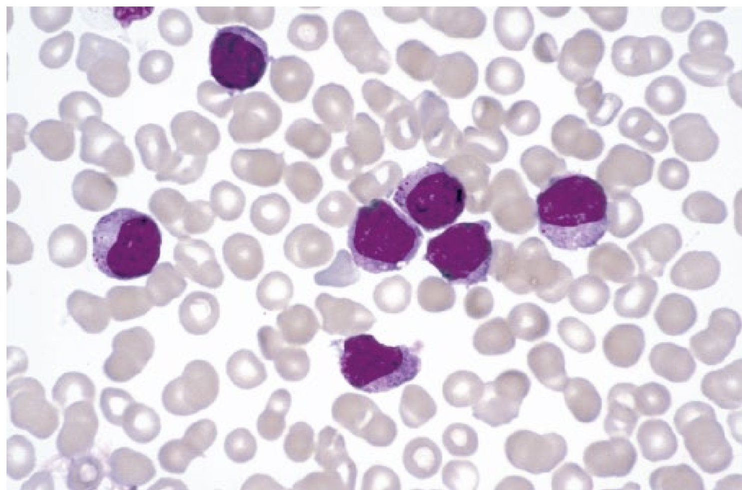


Fig. 7.42 PB film in natural killer-like large granular T-cell lymphoma. The cells are larger and more pleomorphic than in the typical form of large granular T-cell leukaemia and expressed not only CD3 but also four NK markers: CD11b, CD16, CD56 and CD57. (With thanks to Dr Vikas Gupta and colleagues, Southend, with permission of the *British Journal of Haematology*.)

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) [352].

Rheumatoid factor and antinuclear antibodies are often detectable and there is usually a polyclonal increase in immunoglobulins [352].

Immunophenotype

The immunophenotype resembles that of a normal large granular T lymphocyte but differs from it in significant respects [65,366–368]. 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 [368,369]. There is no expression of TdT or CD1 and not usually of CD4 (see Table 7.17). In a minority of patients there is expression of CD4 but not CD8 [370]. Occasionally there are two populations of CD8-positive and CD8-negative cells [371]. 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 [370]. In a study of 12 cases with expression of TCRγδ, eight were CD4−CD8− and four were CD4−CD8+ [372]. Patients with a TCRγδ+CD4−CD8− immunophenotype usually lack peripheral blood involvement but the marrow is infiltrated, often with an intrasinusoidal pattern [373]. CD94 and CD161, both expressed on only a minority of normal T cells, are expressed in around half of patients [368]. There is expression of cytotoxic granule constituents, for example 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 [368,374]; occasionally two KIR molecules are expressed [374]. In one study CD26, a co-stimulatory molecule with CD45 for T-cell activation, was not expressed [369] whereas in another study CD26 was expressed in six of nine patients, expression correlating with severe neutropenia and susceptibility to infection [375]. One case has also been reported showing a mixed T and B immunophenotype [376].

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 [377–379]. There may also be intravascular infiltration, both sinusoidal and intracapillary [368], the latter leading to a linear array of neoplastic cells. There may be reactive lymphoid nodules, containing B cells and CD8-positive T cells and lacking clonal T cells [368,380]. Some cases have plasmacytosis [379]. 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 [381]. Megakaryocytes are virtually absent in patients with amegakaryocytic thrombocytopenic purpura [382]. A significant minority of patients show trilineage myelodysplasia. Reticulin deposition is often increased [380].

Splenic infiltration is in the red pulp, sometimes with an associated plasma cell infiltrate [194].

Cytogenetic and molecular genetic analysis

A number of clonal cytogenetic abnormalities, including trisomy of 3, 8, and 14, 5q−, and inversions of 12p and 14q [356], have been described but no consistent association has been recognized and most patients have a normal karyotype. 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) [383]. Rearrangement at TCR loci is usually demonstrable. This is most often rearrangement of TRB and TRG loci but occasionally it is the TRG locus alone. *STAT3* is mutated in 40% of patients, particularly in patients with rheumatoid arthritis or neutropenia [384]. Uncommonly there is a *STAT5B* mutation, which may be associated with a more aggressive clinical course [385]. Gene expression microarray analysis has revealed six genes that are expressed in T-cell large granular lymphocyte leukaemia but not in normal LGL [386].

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 [368]. 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 [368]. In the 2016 revision of the WHO classification this provisional entity requires the presence of at least 2×10^9/l NK cells persisting for more than 6 months and not explained by any primary disease [387].

Clinical, haematological and cytological features

There are sometimes associated autoimmune conditions or neutropenia [352,388]. Hepatomegaly and splenomegaly are uncommon and the condition shows little tendency to progress [388]. Vasculitis and neuropathy have been reported [356]. Cytologically the neoplastic cells resemble normal LGL. Bone marrow infiltration is interstitial and intrasinusoidal [387].

Immunophenotype

Neoplastic cells do not express CD3 but may express cytoplasmic CD3ε. Expression of CD2, CD7 and CD57 may be weak or absent [387]. 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% [364]. 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 [364]. CD158 expression may be lacking.

Cytogenetic and molecular genetic analysis

The karyotype is usually normal. *STAT3* mutation is present in about a third of patients [389].

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 7.18 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 [390]. 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 [391,392] and the condition may evolve from chronic active EBV infection [390]. EBV-negative cases may evolve from chronic lymphoproliferative disorder of NK cells [390].

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) [352]. Gastrointestinal and central nervous system infiltration and serous effusions can occur. There is a marked male preponderance [356]. The disease shows aggressive clinical behaviour [393,394], is highly resistant to therapy and has a poor prognosis with many patients surviving less than 2 months [352,392]. Death is usually from multiorgan failure with coagulopathy [352] and sometimes a haemophagocytic syndrome.

The peripheral blood shows a variable increase in LGL [392]. 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 [352,392] (Fig. 7.43a). 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 LGL leukaemia [352].

The bone marrow aspirate shows a variable degree of infiltration by cells similar to those in the peripheral blood (Fig. 7.43b). Increased macrophages and haemophagocytosis are often prominent [392,395].

Immunophenotype

The immunophenotype resembles that of a normal NK cell (see Table 7.17) 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 [65,366,396,397].

Histology

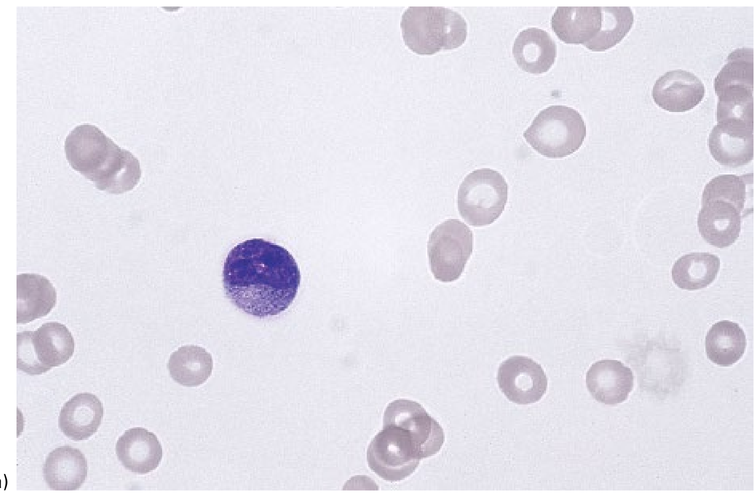
On trephine biopsy histology there is a variable degree of bone marrow infiltration; the pattern of infiltration may be diffuse, interstitial or angiocentric. There is a monomorphic infiltrate

Table 7.18 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).

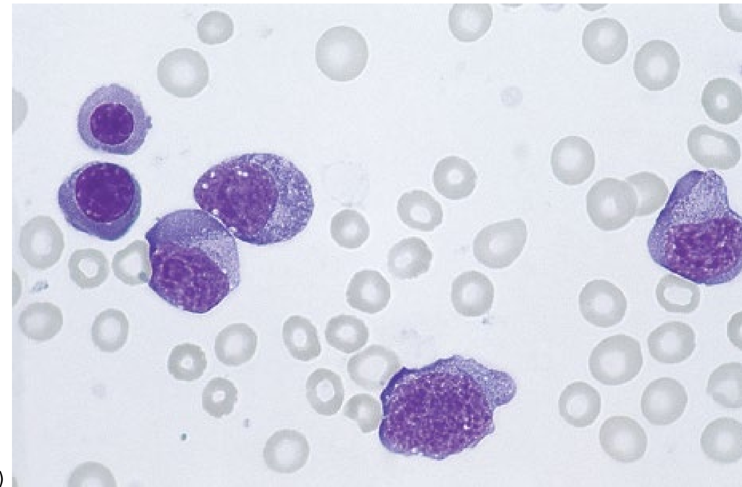
Disease	Cytology	Epidemiology	Rheumatoid arthritis and other autoimmune phenomena	Usual immunophenotype	Clinical course
T-cell large granular lymphocyte leukaemia	Large granular lymphocytes	No EBV association	Yes	CD2+CD3+CD4−, CD8+ CD57+, TCRαβ+, CD11b, CD16 and CD56 variable*, granzyme B+, perforin+ 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 cell; TIA, 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.



(a)



(b)

Fig. 7.43 PB and BM in aggressive NK cell leukaemia: (a) the PB shows a medium-sized lymphoid cell with azurophilic granules while (b), the BM, shows infiltration by medium-sized, granular lymphoid cells, some of which are nucleolated. MGG ×100. (With thanks to Dr A. Martin Noya, Seville.)

of medium-sized cells with round nuclei and condensed chromatin [392]. 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 [352].

Cytogenetic and molecular genetic analysis

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 [265,398]. TCR loci are not rearranged. In most cases, EBV early RNA (EBER) can be detected by *in situ* hybridization [392] with a single episomal form being present [390].

Extranodal NK/T-cell lymphoma, nasal type

This is an angiocentric, angiodestructive EBV-positive lymphoma that is more common in the Far East.

Clinical, haematological and cytological features

The typical clinical presentation is with disease of the nasal cavity, sinuses and adjacent structures. Bone marrow involvement is uncommon

and may be subtle. The infiltration is interstitial and best detected with CD56 immunohistochemistry and *in situ* hybridization for EBV-encoded RNA [399]. Peripheral blood dissemination is rare. Cytological features are variable from patient to patient. Cells may be pleomorphic with cytoplasmic basophilia, lobated nuclei and azurophilic granules [400]. A complicating haemophagocytic syndrome can occur.

Immunophenotype

In most cases the phenotype is that of an NK cell but occasionally it is that of a cytotoxic T cell. The immunophenotype is similar to that of aggressive NK-cell leukaemia except that CD16 is often positive in the latter [401].

Prognosis

The prognosis is often poor and may be worse in those with bone marrow infiltration.

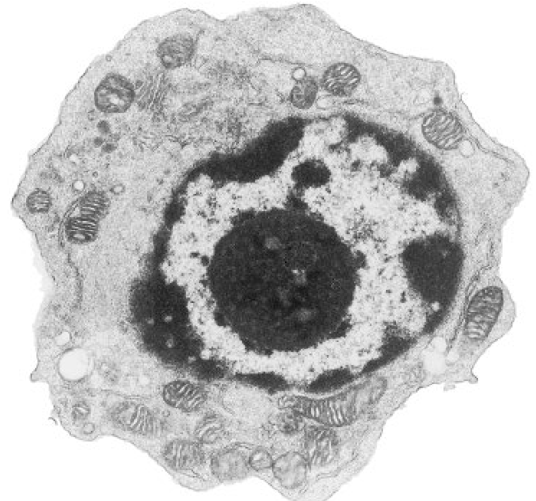


Fig. 7.44 Ultrastructural examination in T-lineage prolymphocytic leukaemia (T-PLL) showing a regular nuclear outline, a prominent nucleolus and relatively abundant cytoplasm with a well-developed Golgi zone, rough endoplasmic reticulum and numerous mitochondria. (With thanks to Dr Estella Matutes, Barcelona.)

T-cell prolymphocytic leukaemia

The FAB group proposed the term T-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 [358]. Ataxia telangiectasia and the Nijmegen breakage syndrome predispose to the development of this type of leukaemia. Cases of T-PLL resemble B-PLL in most often presenting with marked splenomegaly and a high WBC [4,402,403]. 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 [404]. Sometimes the disease course is biphasic with a period of disease stability being followed by rapid progression [405]. A single patient has been reported in whom a slow and complete spontaneous remission occurred [406].

The WBC is moderately to greatly elevated and in about a third of patients there is anaemia and thrombocytopenia [358]. 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. Ultrastructural examination shows the prominent nucleolus very clearly (Fig. 7.44). There may be a minor population of cells with polylobated nuclei or of Sézary-like cells. In other patients cells are smaller with a higher nucleocytoplasmic ratio and a less readily detectable nucleolus (Fig. 7.45). In some patients the nucleus is more convoluted or flower shaped (Fig. 7.46). Because

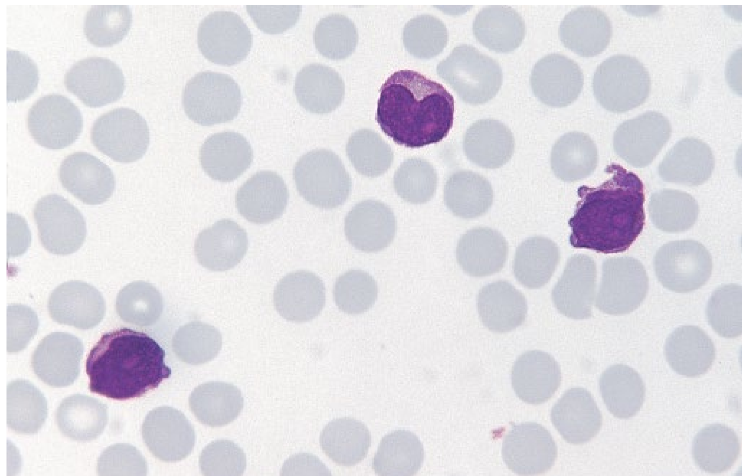


Fig. 7.45 PB film in T-PLL. In this case the nuclei are more irregular and the nucleoli are less conspicuous than in B-PLL. MGG ×100.

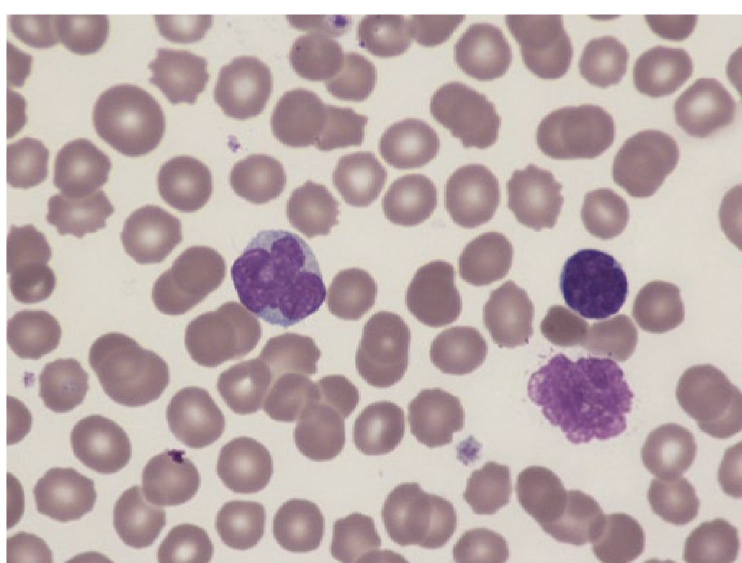


Fig. 7.46 PB film in T-PLL showing a leukaemic cell with a flower-shaped nucleus. The lobulation of the nucleus is also apparent in the smear cell.

the densely condensed chromatin can obscure the nucleolus, it may be more apparent in any smear cells that are present. 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 [91] but nevertheless this is a common feature that therefore suggests this diagnosis. In about 5% of patients, cells have cerebriform nuclei [407]. In a small minority of patients multilobulated cells ('flower cells') dominate [404].

Immunophenotype

In most cases of T-PLL there is expression of CD2, CD3, CD5, CD7, CD4 and TCRαβ

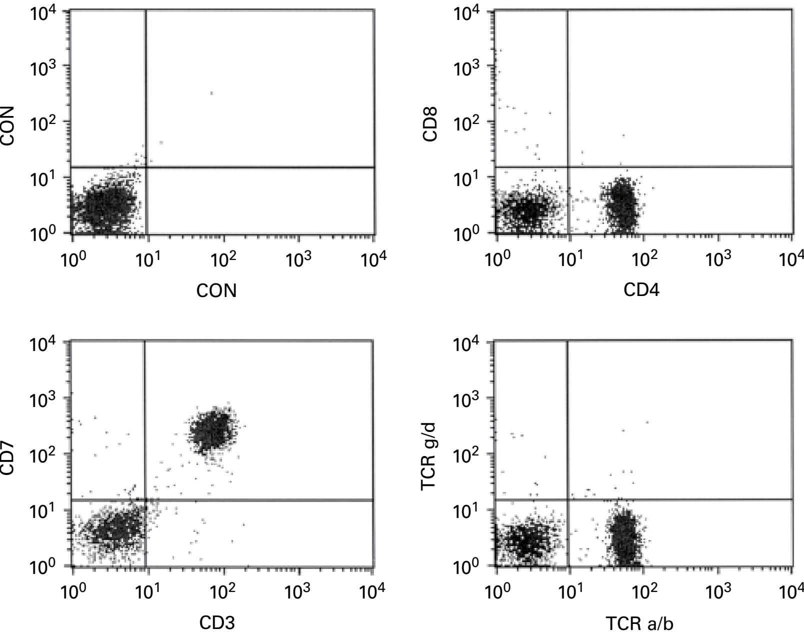


Fig. 7.47 Flow cytometric immunophenotyping in T-PLL showing characteristic positivity for CD3, CD4 and CD7. T-cell receptor (TCR) αβ was expressed but TCRγδ was not. CON, control. (With thanks to Dr Estella Matutes and Mr Ricardo Morilla.)

whereas CD8 is not expressed [4] (see Table 7.17). 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 [369]; about 20% of cases fail to express surface membrane CD3 and TCRαβ [358]. CD7 positivity helps to distinguish T-PLL from other disorders of mature T cells (Fig. 7.47). CD52 expression is usually strong [407]. In about a fifth of patients there is failure of expression of CD26 [370], whereas in the majority of patients it is uniformly expressed (in contrast to the pattern in normal T lymphocytes when only a proportion of cells express CD26). A minority of cases are CD25 positive with expression being weak. A significant minority of cases express CD117 [408]. Some cases, particularly those with aggressive disease, express CD38 [404]. The phenotype CD45R0+CD45RA− also correlates with aggressive disease [404].

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 [407]. Cutaneous infiltration is in the dermis, particularly around vessels and skin appendages. TP53 may be overexpressed. Nuclear and cytoplasmic *TCL1A* and *TCL1B* expression can be detected by immunohistochemistry in the majority of patients and can be used for the detection of residual disease after treatment [370,409]. Strong expression of *TCL1* and *AKT* is prognostically adverse [407].

Cytogenetic and molecular genetic analysis

About three-quarters of cases of T-PLL show either inv(14)(q11.2q32.1) or t(14;14)(q11.2;q32.1) [61,410]. These chromosomal rearrangements involve the TRA locus at 14q11.2 and two oncogenes, *TCL1A* and *TCL1B*, at 14q32.1. *TCL1A* and *TCL1B* are dysregulated and, when

overexpressed, inhibit apoptosis. A less common translocation is t(X;14)(q28;q11.2) in which the *MTCP1* gene at Xq28 is brought into proximity to the TRA locus. Dysregulation of *MTCP1* can also result from proximity of this gene to the TRB locus as a result of t(X;7)(q28;q34) [411]. Further uncommon but recurring translocations include t(11;14)(q21;q32.1) [411] and t(7;14)(q35;q32.1), the latter dysregulating *TCL1A* by proximity to the TRB locus. 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 [412], deletions of the long arms of chromosomes 6 and 11, and translocations with a breakpoint at 11q22.3, 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* [413]. Deletions of 12p13.1 occur and lead to haploinsufficiency of *CDKN1B* [414]. Abnormalities of chromosome 17 involving *TP53* occur in about a quarter of patients [407].

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).

Molecular abnormalities include largely mutually exclusive mutations in *IL2RG*, *JAK1*, *JAK3* or *STATB*, all leading to STAT5 activation, and found in 76% of 50 patients [415]. *EZH2*, *BCOR*, *FBXW10* and *CHEK2* may also be mutated.

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) [4,416,417] occurs in adults who are carriers of the retrovirus, human T-cell lymphotropic virus 1 (HTLV-1); serology for HTLV-1 is positive and proviral DNA is clonally integrated into the DNA of the neoplastic cells. ATLL 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, North Africa, South America (Brazil, Chile, Colombia), Taiwan, Australia (aboriginal population) and a number of other countries. The lifetime risk of ATLL in carriers of the virus has been estimated at 3–5% (5–7% for men and 2–4% for women) [418].

Smouldering and chronic forms of ATLL should also be recognized; suggested criteria are shown in Table 7.19 [419]. Chronic ATLL can be further divided into unfavourable and favourable on the basis of LDH or blood urea above normal or albumin below normal [418]. Disease progression is not inevitable in patients with smouldering or chronic ATLL. It has been found that in HTLV-1-infected patients, the viral burden in peripheral blood mononuclear cells is predictive of progression to acute ATLL rather than whether the peripheral blood criteria for a diagnosis of smouldering ATLL are met [420]. 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 [421]. A pre-leukaemic stage of ATLL in which there is monoclonal proliferation of T lymphocytes is recognized, with about 40% of such individuals progressing to ATLL [422].

Rare cases of ATLL have been related to HTLV-2 rather than HTLV-1 [409].

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

Table 7.19 Subclassification of adult T-cell leukaemia/lymphoma (ATLL) [419].

Category	Peripheral blood lymphocytes	Tissue infiltration	Biochemistry
Smouldering ATLL	Lymphocyte count <4×10^9/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 >4×10^9/l and T lymphocytes >3.5×10^9/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 <4×10^9/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.

lesions (Figs 7.48 and 7.49), hypercalcaemia and circulating lymphoma cells. Skin lesions are in areas exposed to light. Bone lesions are lytic. Some patients have hepatomegaly or splenomegaly or infiltration of other organs. Systemic symptoms are usual. 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 and candidiasis, hyperinfection with *Strongyloides stercoralis* or cytomegalovirus infection. Prognosis is generally poor except for some patients with chronic or smouldering ATLL. A minority of patients suffer from other HTLV-1-related disorders such as HTLV-1-associated myelopathy or uveitis.

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 (Figs 7.50 and 7.51). 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 resemble

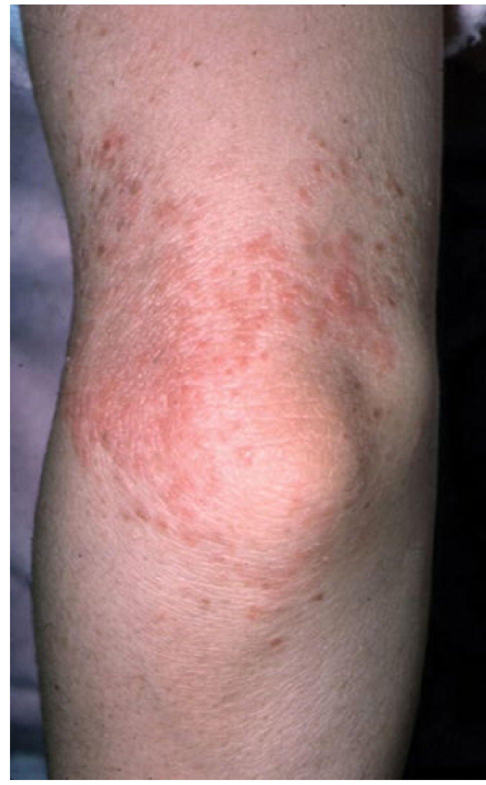


Fig. 7.48 Clinical photograph showing skin lesions in a Japanese woman with adult T-cell leukaemia/lymphoma (ATLL).

Sézary cells, while many are polylobated, with some nuclei resembling cloverleaves or flowers. Some cases have atypical cytological features – LGL in one case [423] and bizarre giant cells resembling Hodgkin cells in others [424].

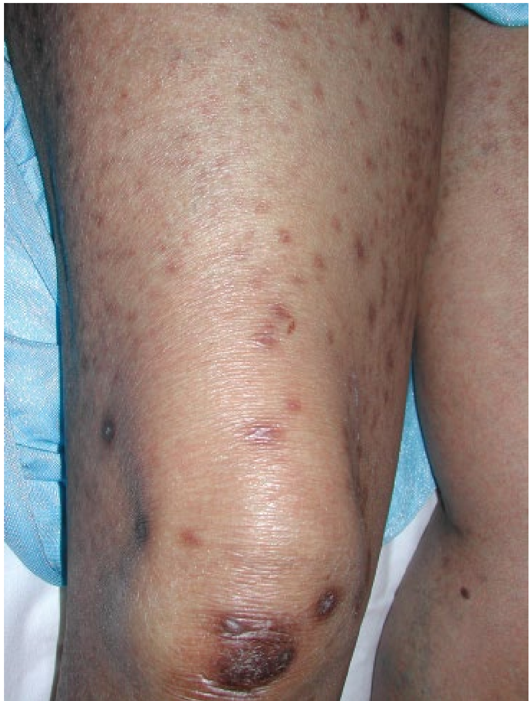


Fig. 7.49 Clinical photograph showing skin lesions in an Afro-Caribbean woman with ATLL.

Cells in serous effusions have similar characteristics (Fig. 7.52).

Some patients are anaemic and some have neutrophilia or eosinophilia. The neutrophilia may be marked, for example a neutrophil count of 16–55 × 10^9/l, and in some cases is attributable to secretion of granulocyte colony-stimulating factor (G-CSF) by neoplastic cells [425].

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 [426]. Among chronic cases, a higher proportion of CLL-like cells is indicative of a better prognosis [426]. Occasional cases of ATLL have been treated by bone marrow transplantation, and in one patient transplanted from an HTLV-1-positive donor, molecular analysis showed that apparent recurrence of leukaemia 4 months after transplantation was of donor origin [427].

Immunophenotype

Adult T-cell leukaemia/lymphoma cells usually express CD2, CD3, CD5, CD4, CD25 and HLA-DR [4,65] (see Table 7.17). A minority of cases are positive for CD7 or CD8 (CD4−CD8+ or CD4+CD8+). In a Japanese study of 36 patients,

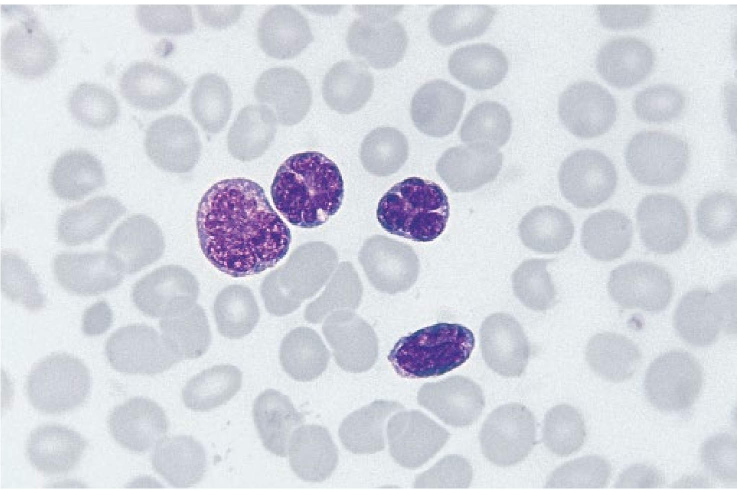


Fig. 7.50 PB film in ATLL. Cells are pleomorphic with polylobulated nuclei, one of which resembles a cloverleaf. MGG ×100.

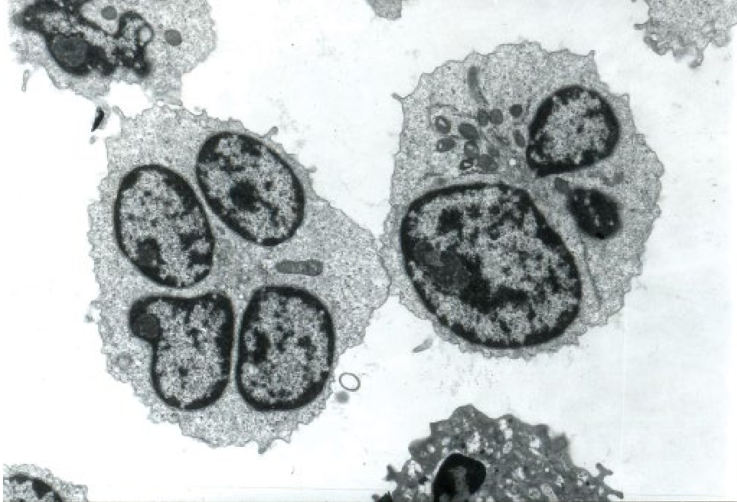


Fig. 7.51 Ultrastructural examination in ATLL showing the multiple lobes of the flower-shaped nuclei. (With thanks to the late Professor David Galton.)

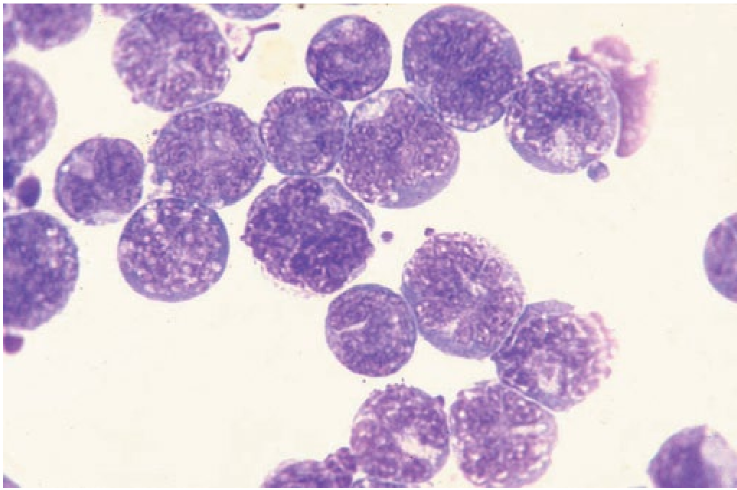


Fig. 7.52 Ascitic fluid of a patient with ATLL showing pleomorphic cells with lobulated nuclei and basophilic cytoplasm. MGG ×100.

CD7 was expressed in 36% of patients, cells were CD4+CD8− in 71% of patients, CD4+CD8+ in 25% and CD8+ in one patient [428]. Expression of CD3 is considerably weaker than on normal T lymphocytes, and CD7, when expressed, is also weaker than on normal cells [369]. Positivity for CD25 helps to distinguish ATLL from other T-cell disorders, which are usually CD25 negative. However, it should be noted that CD25 may not be expressed and it can be expressed on a large proportion of T cells in HTLV-1-positive individuals without ATLL. In the above cited Japanese study [428], only 53% of cases showed CD25 expression by flow cytometry whereas 94% were positive by immunohistochemistry. Other activation markers, such as CD38, may be expressed. CD26 expression is reduced [429]. An atypical immunophenotype, that is other than CD4+CD8−, correlates with a worse prognosis [430].

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 [431]. Lymph nodes show diffuse infiltration, either paracortical or effacing nodal architecture. Neoplastic cells are very pleomorphic 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 [432] but not CD7. There may be expression of CCR4 or FOXP3 [433].

Cytogenetic and molecular genetic analysis

The karyotype is usually abnormal. A variety of chromosomal abnormalities have been described, most commonly trisomy 3, trisomy 7, trisomy 12, trisomy 21, del(6q) and rearrangements with breakpoints at 7p14-15, 14q11-13 or 14q32 [434]. 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*, *CDKN2B* and *TP53*, may be found in the acute and lymphomatous forms of ATLL. Many other genes, including *CCR4*, also show recurrent mutations.

HTLV-1 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 [435]. Integration of defective HTLV-1 is also associated with more aggressive disease [436].

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-1 infection. The presence of intraepidermal lymphoma cells is a recognized feature and should not lead to misdiagnosis of Sézary syndrome.

Sézary syndrome

Sézary syndrome is a cutaneous T-cell lymphoma with circulating neoplastic cells and lymphadenopathy. The 2016 revision of the WHO classification requires, for diagnosis: (i) TCR gene rearrangement and (ii) an absolute Sézary cell count of at least 1.0×10^9/l, or an expanded CD4+ T-cell population resulting in a CD4/CD8 ratio of more than 10, or loss of one or more T-cell antigens (typically CD7 or CD26) [437]. Sézary syndrome can be viewed as an erythrodermic variant of the related mycosis fungoides, but in the WHO-EORTC (European Organisation for Research and Treatment of Cancer) classification of primary cutaneous lymphomas and in the WHO classification they are regarded as separate entities on the basis of a different cell of origin as well as different clinical characteristics [437,438]. Sézary syndrome has a much shorter clinical course than mycosis fungoides.

Clinical, haematological and cytological features

Sézary syndrome is characterized by pruritus and a generalized exfoliative or infiltrative erythroderma, the cutaneous manifestations being the result of infiltration (Fig. 7.53). There may be hair loss, eversion of the eyelids and dystrophic nails.

Neoplastic cells may be either large or small, and one or other form usually predominates in an individual patient [4]. 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. 7.54). Small Sézary cells are similar in size to a normal small lymphocyte, with a high nucleocytoplasmic ratio and a dense

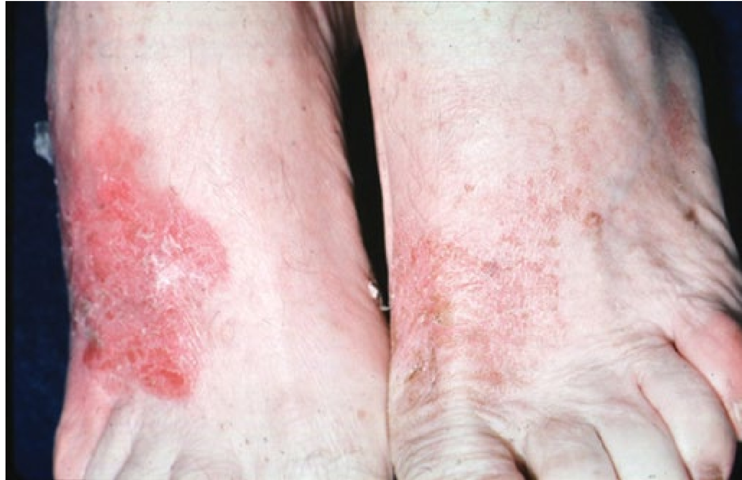


Fig. 7.53 Clinical photograph showing skin lesions of Sézary syndrome.

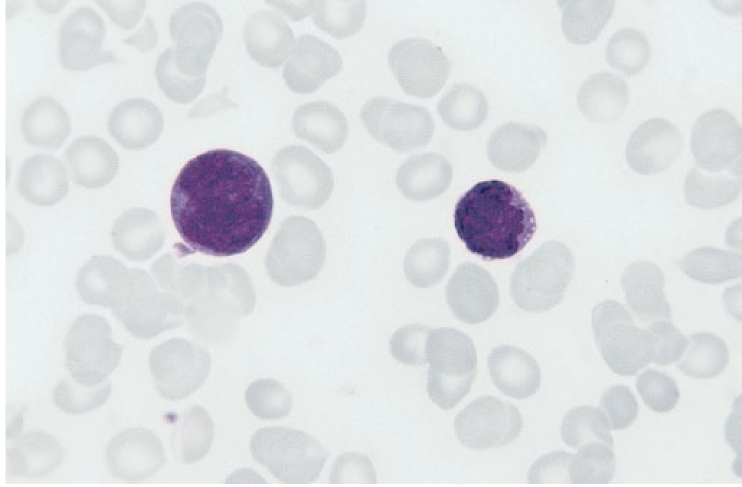


Fig. 7.54 PB film in large cell variant of Sézary syndrome. Both cells have convoluted nuclei; the smaller one has inconspicuous cytoplasmic vacuoles. MGG ×100.

hyperchromatic nucleus with a grooved surface and no visible nucleolus (Fig. 7.55). 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 [439]. 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. 7.56).

The Hb and platelet count are usually normal. There may be a reactive eosinophilia.

Immunophenotype

On flow cytometry, Sézary cells show high forward and high side scatter. The characteristic immunophenotype is expression of CD2, CD3, CD4, CD5 and CD279 with usually no expression of CD7, CD8 or CD25 [4,437,440] (see Table 7.17). Both CD8 [438] and CD25 [441] 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 [409]. A half to three-quarters of cases are CD7 negative [358].

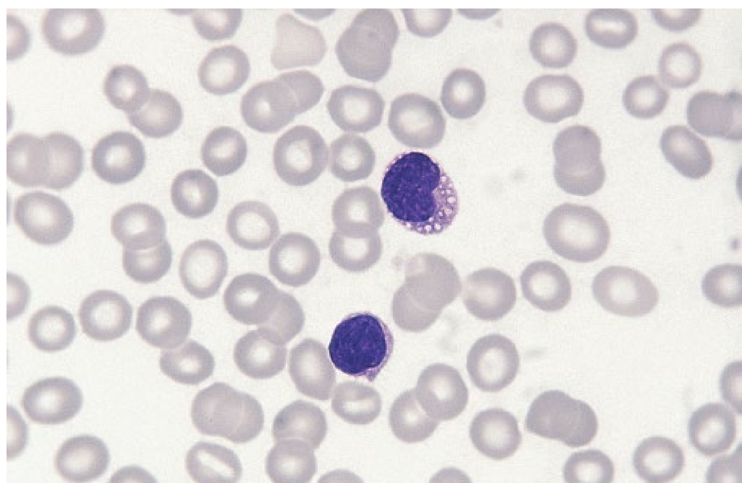


Fig. 7.55 PB film in small cell variant of Sézary syndrome. The nuclei show surface grooves and one cell has a partial circle of vacuoles around the nucleus. MGG ×100.

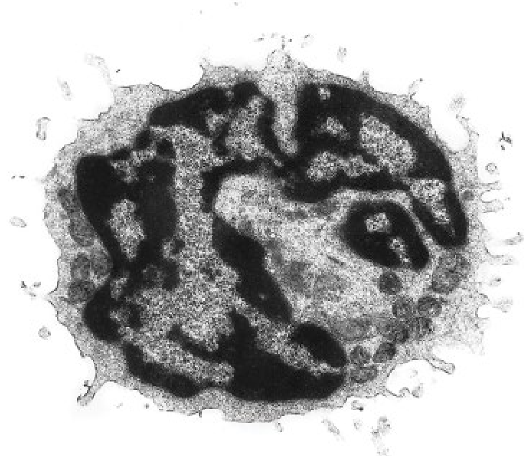


Fig. 7.56 Ultrastructural examination in Sézary syndrome showing the characteristic highly irregular nuclear outline. (By courtesy of Dr Estella Matutes, reproduced with permission from Bain *et al.* 2010 [8].)

TCRαβ is expressed [370]. Lack of expression of CD26 is usual [370] whereas about three-quarters of normal CD4-positive T cells express CD26. Quantification of CD7-negative and CD26-negative T cells has been recommended for both diagnosis and prognostication [442]. CCR4, CCR7 and CD103 (cutaneous lymphocyte antigen) are expressed [437,443]. CD164 is upregulated [444]. Clonality can be demonstrated using antibodies directed at the variable region of the β chain.

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 [445]; (ii) expression of CD158k [446]; and (iii) absent or weak expression of CD26, usually associated with weak expression of CD4 [447]. Detection of more than 11% of CD4+ CD7− cells by flow cytometry has been found useful in confirming the diagnosis of Sézary syndrome [440,441]. Diagnostic criteria for peripheral blood involvement, incorporating immunophenotyping and other data, have been proposed by the International Society for Cutaneous Lymphomas [447]. These are summarized in Table 7.20 [448].

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 but are not always present and are not pathognomonic since they have now also been observed in a number of cases of ATLL. Lymph node infiltration may be

Table 7.20 International Society for Cutaneous Lymphomas criteria for diagnosis of Sézary syndrome [448].

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 a T-cell lymphoma on biopsy or as shown below
Immunophenotyping	CD4 : CD8 ≥10 : 1 as a result of increased CD3+CD4+ lymphocytes
	OR
	Aberrant lack of expression of pan-T markers CD2, CD3, CD4 or CD5
	OR
	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

paracortical or lymph nodes may be effaced. CD45RO is expressed [446].

Cytogenetic and molecular genetic analysis

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 abnormality based on the detection of aneuploidy by flow cytometry has been found useful in diagnosis [449].

No specific molecular genetic abnormality has been demonstrated but numerous genes show recurrent mutation. Loss of function of *ARID1A* is common [437]. The *JUNB* oncogene may be amplified and there may be inactivation of *TP53* and *CDKN2A* [437]. Demonstration of rearrangement at the TCR loci is useful in confirming the diagnosis and has been found to be more sensitive in the detection of peripheral blood involvement than morphometric analysis [443].

Prognosis

Visceral involvement is adverse [437]. An increasing number of circulating lymphoma cells correlates with worse prognosis [450]. The lymphocyte count and the percentage of Sézary cells have been found to be predictive of response to extracorporeal photopheresis [451].

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 loci rearrangement should be a prerequisite for diagnosis [443]. Alternatively, other criteria proposed by the International Society for Cutaneous Lymphomas (Table 7.20) 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 [358,452,453]. Consideration of the immunophenotype – CD7 expression – and the cytogenetic features – usually complex cytogenetic abnormalities including inv(14)(q11.2q32) and possibly idic(8)(p11) – permit the correct diagnosis [452].

Mycosis fungoides

Mycosis fungoides is an epidermotropic cutaneous T-cell lymphoma [454] characterized clinically by patches, plaques and, eventually, tumours (Fig. 7.57); the tumours may ulcerate. Erythroderma is uncommon and when it occurs is indicative of more generalized disease. Mycosis fungoides usually runs a chronic course but transformation to an aggressive large cell lymphoma, including anaplastic large cell lymphoma,

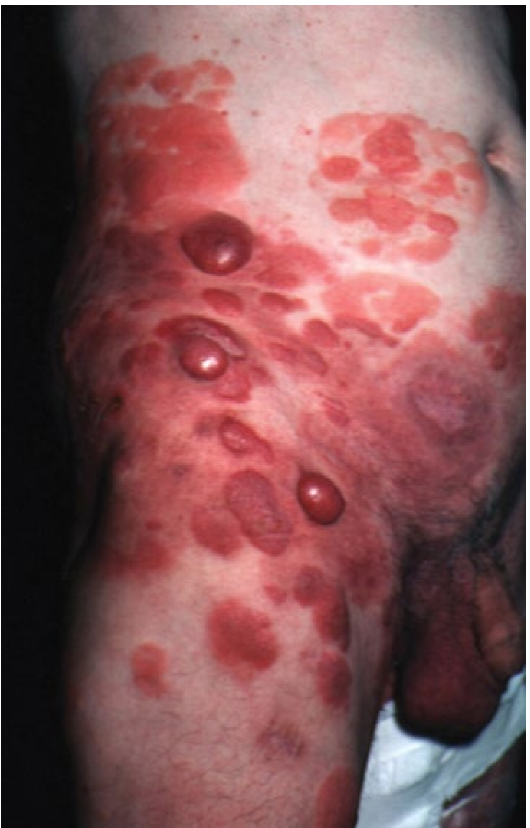


Fig. 7.57 Clinical photograph showing plaque and tumour lesions of mycosis fungoides.

can occur. An aetiological role for occupational exposure to various chemicals is suspected [454].

Clinical, haematological and cytological features

Mycosis fungoides may be restricted to the skin. When the disease becomes more generalized, lymphoma cells similar to those in Sézary syndrome may circulate in the blood. Bone marrow infiltration is rare.

The blood count is usually normal. There may be a reactive eosinophilia.

Immunophenotype

The neoplastic cells usually show expression of CD2, CD3, CD4, CD5, CD103 and TCRβ, with usually no expression of CD7, CD8, CD25, CD26 or TCRγ. A minority of cases show expression of CD8 or TCRγ.

Histology

Epidermotropism is characteristic of mycosis fungoides. Pautrier microabscesses are highly characteristic but are seen in only a minority of patients. In the later stages of the disease, when there is tumour formation, epidermotropism may be lost with tumour being present in both the upper and lower dermis. CD30 may be expressed, in addition to T-cell markers.

Cytogenetic and molecular genetic analysis

Complex karyotypes may be present, particularly with advanced disease [454]. A number of genes show recurrent mutations.

Prognosis

Prognostic factors include advanced stage, older age, higher LDH and histological transformation [454].

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 [455] and in other immunosuppressed patients [456].

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 [457] (Fig. 7.58). Cytologically, the neoplastic cells cannot be readily distinguished from those of other T-cell lymphomas, with no consistent features being identified. In one reported case the cells resembled virally activated T cells, whereas in another they resembled medium to large lymphoma cells, and in a third patient the neoplastic

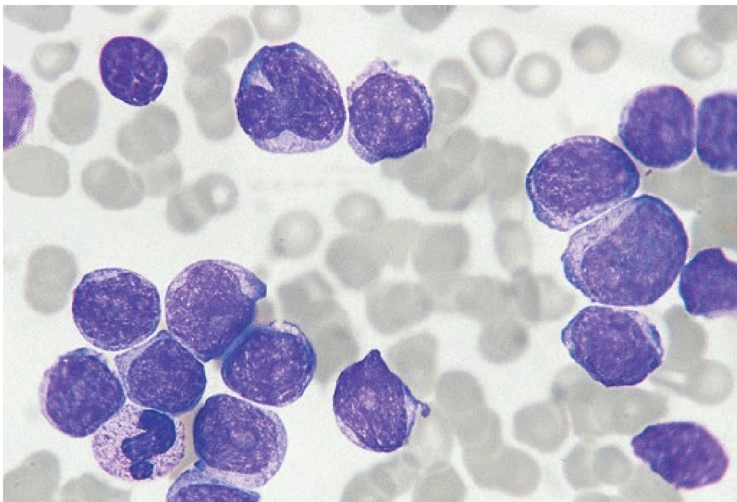


Fig. 7.58 PB film from a patient with hepatosplenic lymphoma in leukaemic phase showing large pleomorphic cells with basophilic cytoplasm. MGG ×100.

cells had convoluted nuclei and a high nucleocytoplasmic ratio [458]. Sometimes neoplastic cells have 'hairy' projections [459]. Erythrophagocytosis by lymphoma cells has been reported [460]. 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 [457]. There may be increased plasma cells and reactive haemophagocytosis.

Immunophenotype

Characteristically there is expression of CD2, CD3, CD7, CD11b and CD56 [457,461]. CD4, CD5 and CD8 are usually not expressed. CD16 is often expressed whereas CD57 is not [265]. 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 [456].

Histology

Early in the disease course, bone marrow infiltration is interstitial and intrasinusoidal; the characteristic intravascular pattern of infiltration may suggest this diagnosis (Fig. 7.59). With advancing disease, there is a more extensive interstitial infiltration. There is typically hyperplasia of myeloid cells. The lymphoma cells express TIA-1 and granzyme M, but usually not granzyme B or perforin [456]. They may express CD158 with CD94 being negative [456].

Cytogenetic and molecular genetic analysis

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 the TRG locus, but not of the TRB locus. *STAT5B* is often mutated [456].

Prognosis

Prognosis is generally poor.

Other T-lineage non-Hodgkin lymphomas

Rarely leukaemia occurs as a manifestation of other 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. In enteropathy-associated T-cell lymphoma, the presentation is

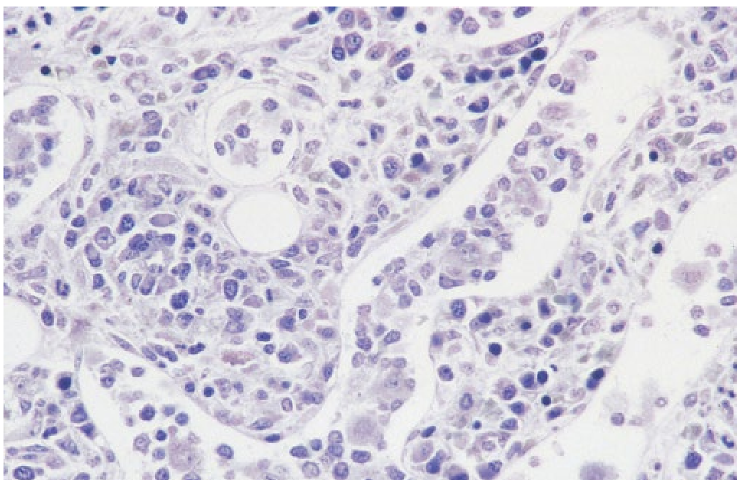


Fig. 7.59 Trephine biopsy section from a patient with hepatosplenic lymphoma in leukaemic phase showing intravascular lymphoma (same patient as Fig. 7.58). Giemsa ×40.

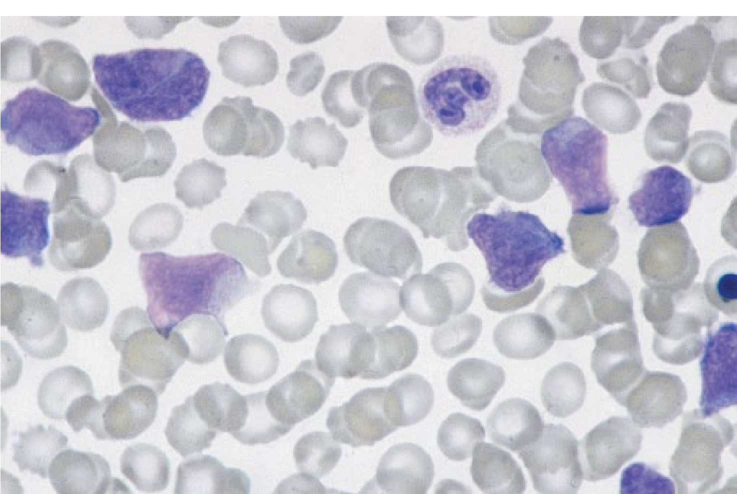


Fig. 7.60 PB film in T-lineage lymphoma. MGG ×100.

with gastrointestinal symptoms, sometimes with a diagnosis of prior coeliac disease and sometimes with a *de novo* presentation with intestinal perforation. The blood count in T-cell lymphoma may show anaemia or thrombocytopenia. 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. 7.60). The neoplastic cells of enteropathy-associated T-cell lymphoma may have azurophilic granules. In a rare case of peripheral blood involvement by angioimmunoblastic T-cell lymphoma, the neoplastic cells were small to medium sized with a high nucleocytoplasmic ration and weakly basophilic cytoplasm [462].

In some patients with a leukaemic phase of ALK-positive anaplastic large cell lymphoma, lymphoma cells are very large and pleomorphic (Fig. 7.61). 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 [463–466]. There is an irregular, hyperchromatic nucleus, which can be lobulated, cerebriform or cloverleafed; azurophilic cytoplasmic granules are

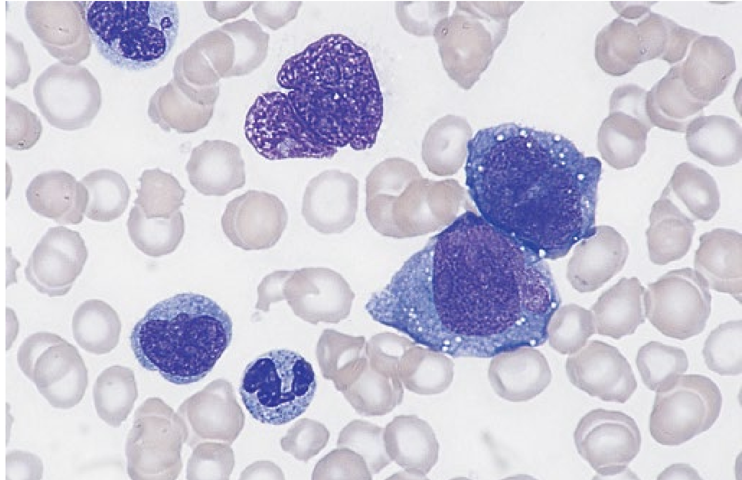


Fig. 7.61 PB in anaplastic large cell lymphoma. The lymphoma cells are very large and very pleomorphic. MGG ×100. (With thanks to Dr David Clark, Grantham.)

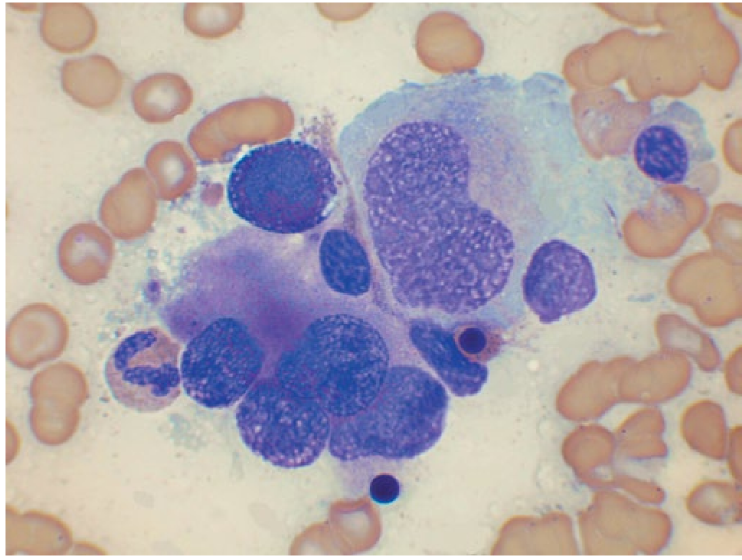


Fig. 7.62 BM aspirate in anaplastic large cell lymphoma. The lymphoma cell (top right) is as large as the megakaryocyte (bottom left). MGG ×100.

sometimes present. Sometimes lymphoma cells with flower-shaped nuclei are prominent [467]. There may be occasional large cells that resemble Reed–Sternberg cells [465]. The prognosis appears to be considerably worse in those patients who present with leukaemic manifestations [466]. Hyperleucocytosis can occur. Bone marrow infiltration may be minimal but cells are distinctive (Fig. 7.62). Presentation of ALK-negative anaplastic lymphoma in leukaemic phase has also been reported; the cells were very large with folded or convoluted nuclei, partially clumped chromatin, multiple distinct nucleoli and a moderate amount of basophilic cytoplasm, sometimes containing fine vacuoles or azurophilic granules [468].

The neoplastic cells of these T-cell lymphomas cannot generally be distinguished from those of B-lineage lymphomas on the basis of cytological features [329] so that immunophenotyping is essential for precise diagnosis.

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 [329]. 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 [469].

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 [465]. In addition they usually express CD30, ALK1 (not normally expressed by haemopoietic or lymphoid cells) and EMA [465], this being more readily demonstrable on immunohistochemistry of trephine biopsy sections. CD38, CD56 and HLA-DR may be expressed, but not CD34. CD26 is uniformly expressed [190]. Myeloid antigens such as CD11b, CD13, CD15 and CD33 may be expressed [265]. Enteropathy-associated T-cell lymphoma also has a specific immunophenotype with expression of CD103 in addition to CD2, CD3 and CD7; typically there is no expression of CD4 or CD8 but some cases show CD8 expression; CD56 is often expressed and sometimes CD30 [190]. Angioimmunoblastic T-cell lymphoma is characterized by expression of CD10 in addition to CD3 (which 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 analysis

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)(p23.2;q35.1) leading to *NPM1-ALK* fusion. The fusion transcript can be detected by RT-PCR and abnormal ALK expression by immunohistochemistry [270]. 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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8

Leukaemia Diagnosis in Resource-Poor Countries

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The gold standard of care is that which achieves the best outcomes with the available resources.

Peter Hesseling

Introduction

No country has unlimited resources to apply to medical care, but problems are particularly acute in low- and middle-income 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 [1]. Which particular haematological neoplasms can be treated effectively will depend on the ability to recognize the condition, the availability of skilled nursing and medical care, the existence of an adequate blood transfusion service, and the 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 [2]. However, modern technology is

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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 myeloid leukaemia (CML). Similarly, flow cytometry for the detection of minimal residual disease (MRD) in acute lymphoblastic leukaemia (ALL), using an abbreviated panel of antibodies, may save lives by avoiding unnecessarily intensive treatment with associated toxicity [3]. 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, for example 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, for instance cytogenetic analysis, to a laboratory in another country.

Considerable improvements in the 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 [4]. Collaborative schemes for acute leukaemia diagnosis have been established: between US centres and Brazil, Nicaragua, El Salvador, Honduras, Guatemala, Mexico and India; between the UK and Ghana and Malawi; and between Nicaragua, Italy and Switzerland [4,5–10]. An international consortium was established in 2005 to improve the diagnosis and management of acute promyelocytic leukaemia (APL) in developing countries, with improved survival being documented in a study involving Brazil, Mexico, Uruguay and Chile [11]; subsequently the scheme was extended also to Paraguay and Peru. The initial step in such collaborations may be standardized diagnostic protocols and management plans, with some of the diagnostic procedures possibly being carried out in a developed country but with progression 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, for example ultralight unmanned aircraft for transport of small specimens within a country has been pioneered in South Africa. A more conventional combination of postage and email can give a turnaround time averaging about 2 weeks [9]. Consultation with experts may also be possible through the internet and email or file-hosting services such as Dropbox (tele-haematology) [10]. 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 adaptations; the captured image can be transmitted through the mobile phone network or by email [12].

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 many developing countries patients may have no medical insurance or social security support and, since they then need to pay for every test, there may be serious financial constraints affecting diagnosis as well as treatment.

The rest of this chapter 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 and has been reviewed in detail in Chapter 1. Many leukaemias can be recognized from clinical context, blood count and cytological 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 (APL), 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 (NSE) such as α-naphthyl acetate esterase (ANAE) 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 ALL it is highly likely that it does represent ALL (Fig. 8.1). 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 (Fig. 8.2). Cytological 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; clinical features might also point strongly to a lymphoid or myeloid lineage (Fig. 8.3).

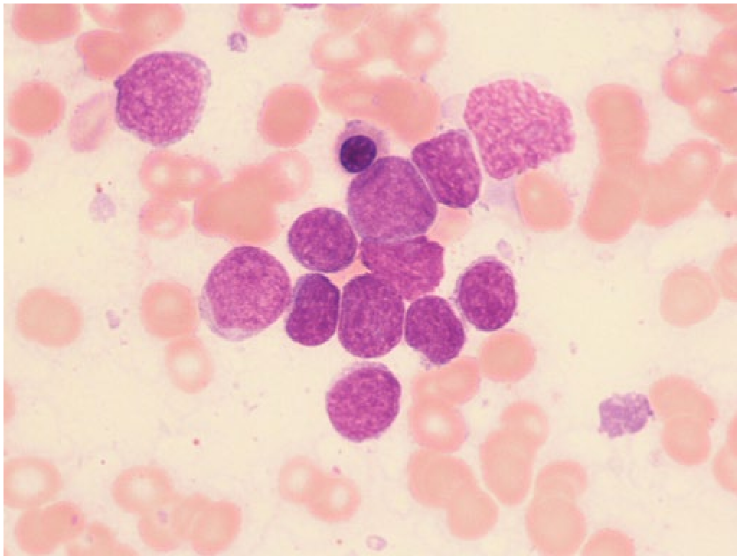


Fig. 8.1 Bone marrow (BM) film of B-lineage acute lymphoblastic leukaemia (B-ALL) showing French–American–British (FAB) L1 cytological features. The small to medium-sized blast cells are regular in shape and have a high nucleocytoplasmic ratio. The larger blast cells have a diffuse chromatin pattern while the small cells show some chromatin condensation. These cytological features make acute myeloid leukaemia (AML) very unlikely and, in the absence of other diagnostic resources, treatment as ALL is appropriate.

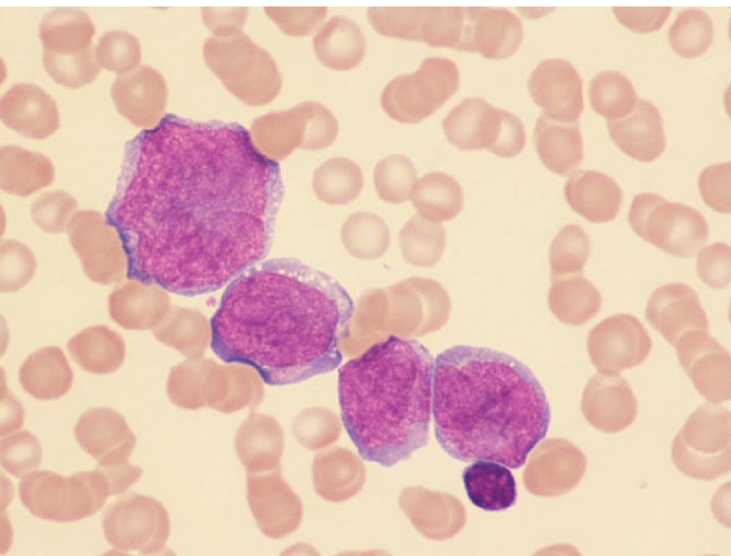


Fig. 8.2 Peripheral blood (PB) film from a case of B-ALL showing L2 cytological features. The blast cells vary in size and are pleomorphic. Some nuclei are lobulated and some show nucleoli of variable size. In the absence of at least cytochemistry, this case cannot be recognized as ALL.

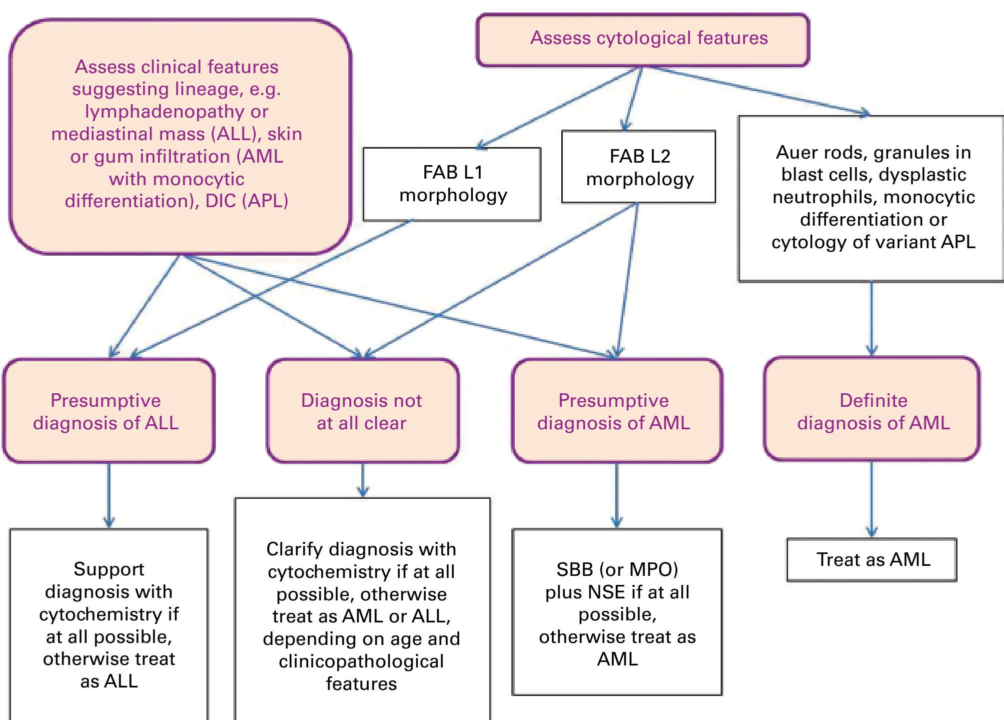


Fig. 8.3 Flow chart illustrating diagnostic pathways in acute leukaemia when immunophenotyping is not available. Abbreviations: ALL, acute lymphoblastic leukaemia; AML, acute myeloid leukaemia; APL, acute promyelocytic leukaemia; DIC, disseminated intravascular coagulation; FAB, French–American–British (classification); MPO, myeloperoxidase; NSE, non-specific esterase; SBB, Sudan black B.

APL comprises up to 25% of cases of AML in Latin America, a much higher prevalence than in developed countries. For this reason, and because of the efficacy of highly specific therapy and the need for intensive support of the associated coagulopathy, its recognition is particularly important. The hypergranular variant can usually be recognized on microscopy with a high degree of reliability. Recognition of the variant hypogranular/micrgranular form is more difficult but is possible with careful attention to cytological details (see Fig. 1.24). The observation of coexisting disseminated intravascular coagulation (DIC) supports the diagnosis. Either diagnosis can be confirmed by molecular analysis in a single centre per country [11].

Consideration should be given to the possibility of flow cytometric 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, for example: 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 is usually possible to recognize that a case of leukaemia represents acute leukaemia by morphology, TdT (and CD34) may be considered inessential. Resource-stratified guidelines for Asia suggest the use of CD19, CD22 or CD79a, CD7 and cytoplasmic CD3, and MPO when the suspected diagnosis is ALL, with CD20 being added when resources permit [13]. 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 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 antilysozyme 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.

When immunophenotyping is not available, cytochemical reactions continue to be informative but must be used with a constant awareness of their lack of specificity. Lymphoblasts show negative reactions for MPO and chloroacetate esterase (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 [14]; these very fine granules probably represent mitochondria. Rare cases of apparent ALL have shown coarse granular positivity with SBB [14,15]. 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.

The block positivity on PAS stain that is characteristic of B-lineage ALL (Fig. 8.4) is seen also, although perhaps less often, in T-lineage ALL.

*CD, Cluster of Differentiation

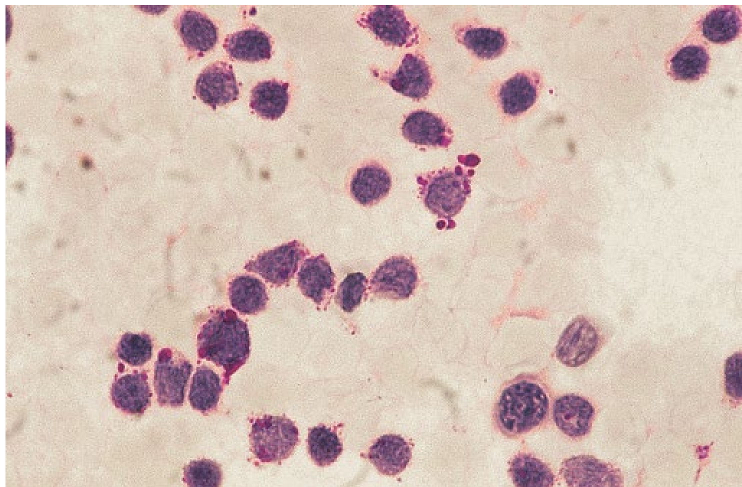


Fig. 8.4 Periodic acid-Schiff (PAS) stain of the bone marrow of a patient with common B-ALL showing block positivity. PAS ×100. (With thanks to Dr Ayed Eden, Southend-on-Sea.)

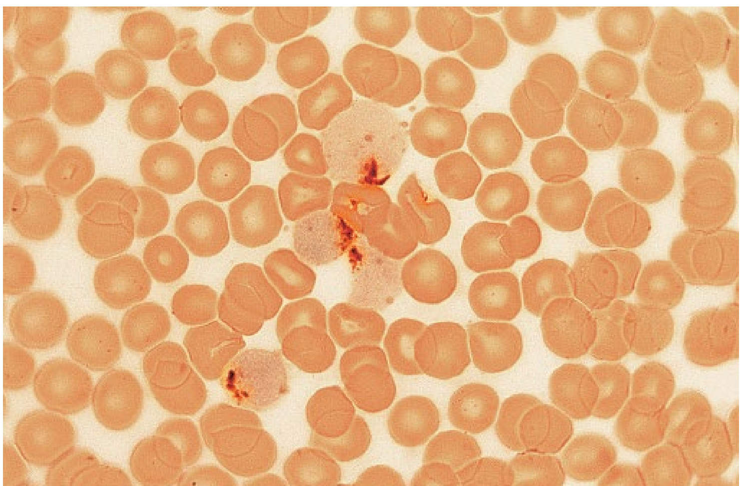


Fig. 8.5 Acid phosphatase stain of the peripheral blood of a patient with T-lineage ALL showing focal positivity. Acid phosphatase reaction ×100.

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. 1.38e).

The presence of strong localized positivity for acid phosphatase is common in T-lineage ALL (Fig. 8.5) 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 FAB M6 AML and may also be seen in M7 AML [16]. In a minority of cases of B-lineage 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 [17]. T lymphoblasts may also have localized coarse granular positivity for NSE, 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.

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 (see Fig. 8.1). Likewise a child with cytological features of L1 ALL, particularly if there is prominent lymphadenopathy, 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 NSE will help to confirm acute monoblastic leukaemia. Resource-stratified guidelines for Asia suggest the use of MPO and NSE when immunophenotyping is not available [13].

If a definite lineage assignment cannot be made in a case of acute leukaemia, a trial of therapy may help, for example chemotherapy appropriate for ALL if the differential diagnosis is between ALL and AML, or a trial of all-*trans*-retinoic acid (ATRA) if APL 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 CML 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 a myelodysplastic syndrome (MDS).

When supplementary techniques are not available it is particularly important to be able to recognize the cytological features of conditions that may involve the bone marrow and simulate a haematological neoplasm, specifically neuroblastoma (Fig. 8.6), which may simulate acute leukaemia, and rhabdomyosarcoma (Fig. 8.7), which may simulate Burkitt lymphoma (Fig. 8.8).

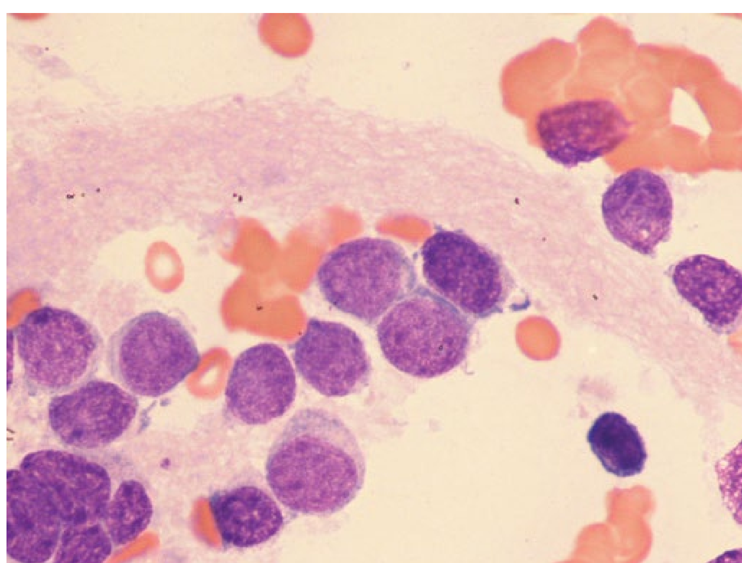
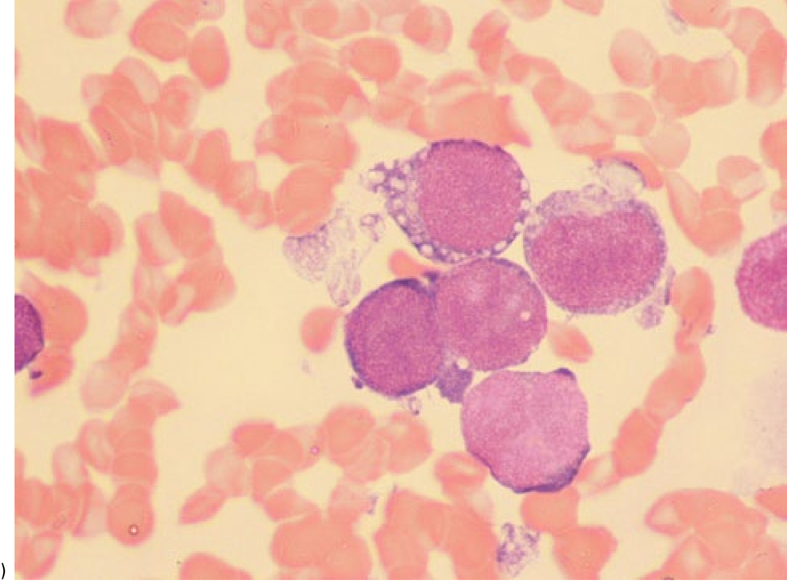


Fig. 8.6 BM aspirate film showing infiltration by neuroblastoma. Although the neoplastic cells have some similarities to lymphoblasts, there are two diagnostic clues: the cohesive clump of cells showing nuclear moulding (bottom left) and the neurofibrillary bundles (top).

(a)



(b)

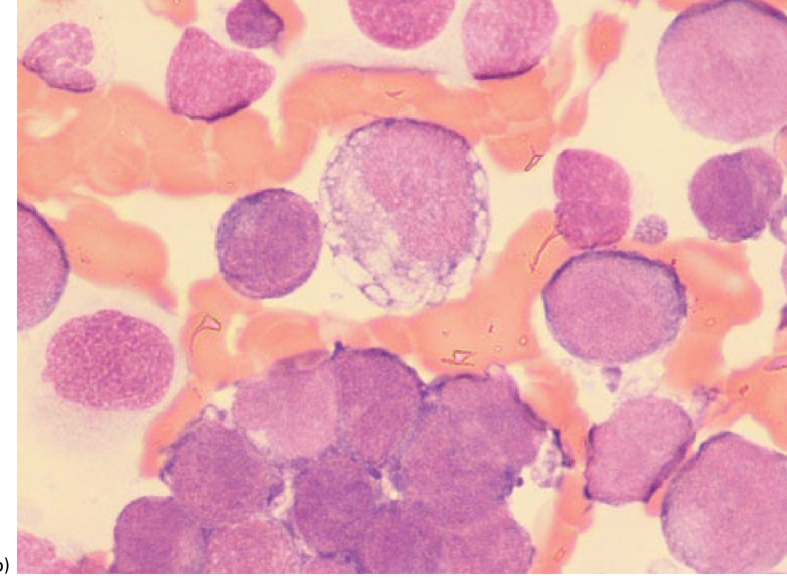


Fig. 8.7 BM aspirate showing infiltration by rhabdomyosarcoma: (a) the neoplastic cells have some similarities to Burkitt lymphoma cells but note the stippled chromatin pattern and the fact that the vacuoles are tending to coalesce, rather than remaining distinct and perfectly round; (b) a cohesive clump of cells (bottom) indicates that this is a non-haemopoietic neoplasm, and the large, irregular coalescing vacuoles (centre top) make clear that this is not Burkitt lymphoma. The vacuoles are PAS-positive glycogen in contrast to the lipid-containing vacuoles of Burkitt lymphoma.

Diagnosis of chronic myeloid leukaemia

Morphology of peripheral blood and a careful differential count, interpreted in the light of the clinical history and physical findings, will identify patients who are highly likely to have CML. A low neutrophil alkaline phosphate score supports the diagnosis but if imatinib is available, a definitive diagnosis is needed. 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. The International Chronic Myeloid Leukemia Foundation (www.cml-foundation.org) facilitates diagnosis and monitoring globally. The Max Foundation (https://themaxfoundation.org) has

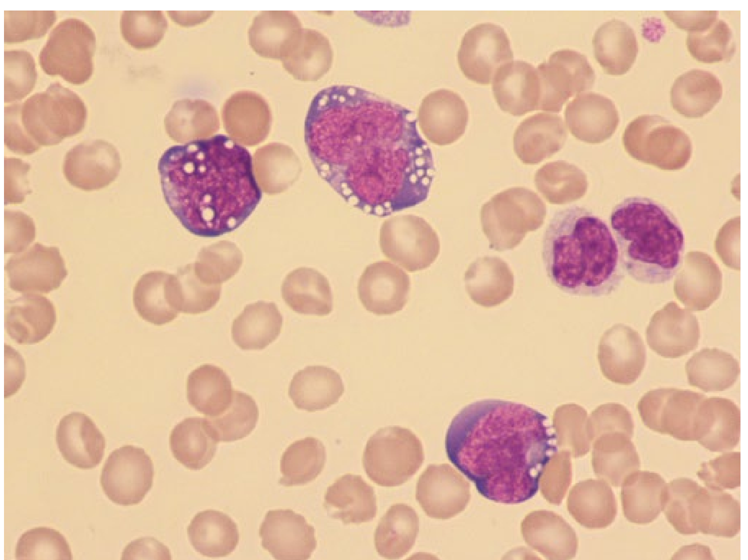


Fig. 8.8 PB film, Burkitt lymphoma, showing strong cytoplasmic basophilia and prominent vacuolation. Note that the irregularly condensed chromatin pattern differs from that of rhabdomyosarcoma and the distinct vacuoles are round.

an agreement with Cepheid for High Burden Developing Countries such that the provision of diagnostic tests for CML (GeneXpert polymerase chain reaction) is subsidized. Together with Novartis, the provision of tyrosine kinase inhibitors for therapy in low- and middle-income countries has been made possible.

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 histology. 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 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. In assessing dysplasia, it is important to be aware of the frequency of dysplasia, particularly of neutrophils, in HIV infection. 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 del(5q), either as an isolated abnormality or with a single additional cytogenetic abnormality other than del(7q) or monosomy 7. 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 CML 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 CML.

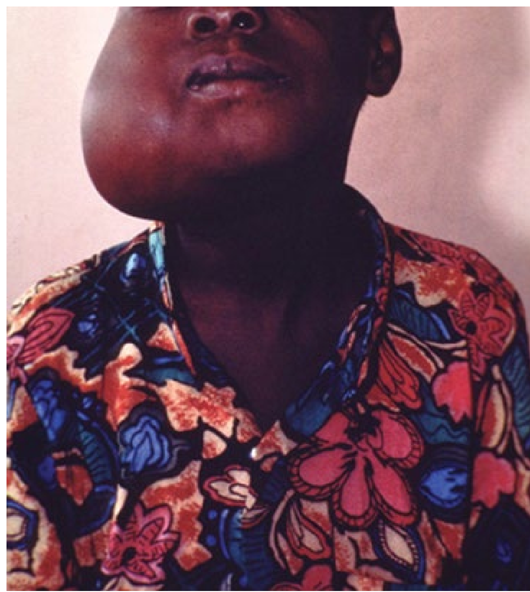


Fig. 8.9 Clinical photograph of a child with endemic Burkitt lymphoma. (With thanks to Dr Fiona Hampton.)

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. Histology 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/or histological features (Fig. 8.9). The peripheral blood and bone marrow are not often involved in endemic Burkitt lymphoma; in this circumstance, a lymph node aspirate or core biopsy can confirm a clinical diagnosis. If a single immunohistochemical stain were to be added for confirmation it would be a stain such as MIB1 for Ki-67 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 (see page 448) 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 moderately effective specific treatments the diagnosis is important. Cytology is often very characteristic (see page 485) and if there is hypercalcaemia the suspicion of this diagnosis is strengthened. Demonstration of seropositivity for the human T-cell lymphotropic virus 1 (HTLV-1) 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 CD3 and usually also 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 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.

Supplementary tests

In many low- and middle-income countries there is a probability of associated infections, which should be identified before treatment of a haematological neoplasm is started. Depending on the geographical area, these may include infection by HIV or hepatitis B, tuberculosis, and malaria or other parasitic infections. Tests of liver and renal function, lactate dehydrogenase, uric acid assay and blood group and antibody screening are indicated. A coagulation screen is vital if APL is suspected.

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 8.1 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

Table 8.1 Websites giving useful information in the diagnosis of leukaemia and related conditions.

Organization	Web address
American Society of Hematology	http://www.hematology.org/ http://imagebank.hematology.org
European LeukemiaNet (site includes an atlas and information on cytogenetics and immunophenotyping)	http://www.leukemia-net.org/content/home/
British Society for Haematology (BSH) (includes an image atlas, and now incorporates the educational component previously available at www.bloodmed.com and the guidelines of the British Committee for Standards in Haematology)	http://www.b-s-h.org.uk/
European Hematology Association	http://www.ehaweb.org/
Atlas of Genetics and Cytogenetics in Haematology and Oncology	http://atlasgeneticsoncology.org/
Bloodline (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
Atlas of Blood Cells and Blood Diseases (T. Vallespí and L. García-Alonso)	www.atlasbloodcells.es

countries. Books and bench aids are available to developing countries at a reduced cost from the WHO. The UK charity, Teaching Aids at Low Cost (http://www.talcuk.org/), provides low-price books to developing countries. Many medical journals are available free of charge to low and low- or middle-income countries. The UK Department of International Development, through the Development Partnerships in Higher Education (DelPHE) programme, supports individuals to visit low- and middle-income countries for teaching and support (https://www.gov.uk/guidance/development-partnerships-in-higher-education-delphe).

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