Two
SPECIAL TECHNIQUES APPLICABLE TO BONE MARROW DIAGNOSIS

Peripheral blood samples, bone marrow aspirates and trephine biopsy specimens are suitable for many diagnostic investigations, in addition to routine microscopy of Romanowsky-stained blood and bone marrow films and haematoxylin and eosin-stained histological sections. Some of these techniques, for example Perls’ stain to demonstrate haemosiderin in a bone marrow aspirate, are so often useful that they are performed routinely, whereas other techniques are applied selectively. This chapter will deal predominantly with special techniques that are applicable to bone marrow aspirates and trephine biopsy sections but reference will be made to the peripheral blood where this is the more appropriate tissue for study.

Bone marrow aspirate films are stained routinely with a Romanowsky stain such as a May–Grünwald–Giemsa (MGG) or a Wright–Giemsa stain. Other diagnostic procedures that may be of use in individual cases include: (i) cytochemistry; (ii) immunophenotyping (by immunocytochemistry or flow cytometry); (iii) cytogenetic and molecular genetic analysis; (iv) ultrastructural examination; (v) culture for micro-organisms; and (vi) culture for assessment of haemopoietic progenitor cells.

In most countries, histological sections cut from bone marrow trephine biopsies are stained routinely with H&E. Most laboratories also use silver stains routinely to demonstrate reticulin and some employ, in addition, a Giemsa stain, a Perls’ stain or both. We recommend the routine use of H&E, Giemsa and reticulin stains. Giemsa staining permits the easy identification of mast cells, facilitates recognition of plasma cells and helps in making a distinction between early erythroid cells and myeloblasts. If a Giemsa stain is not performed routinely, then it is important that it is used whenever necessary for these indications. Other techniques that may be applied to trephine biopsy sections include: (i) a wider range of cytochemical stains; (ii) immunohistochemistry; (iii) cytogenetic and molecular genetic analysis; and (iv) ultrastructural examination.

Cytochemical and histochemical stains

Cytochemical stains on bone marrow aspirates

Perls’ stain for iron

A Perls’ or Prussian blue stain (Figs 2.1 and 2.2) demonstrates haemosiderin in bone marrow macrophages and within erythroblasts. Consequently, it allows assessment of both the amount of iron in reticulo-endothelial stores and the availability of iron to developing erythroblasts.

Assessment of storage iron requires that an adequate number of fragments are obtained. A bone marrow film or squash will contain both intracellular and extracellular iron, the latter being derived from crushed macrophages. It is usual to base assessment of iron stores mainly on intracellular iron since iron stains are prone to artefactual deposits and it can be difficult to distinguish between extracellular iron and artefact. Iron stores may be assessed as normal, decreased or increased, or may be graded as 1+ to 6+ as shown in Table 2.1. Grades of 1+ to 3+ being considered normal. Alternatively, iron stores may be graded as 1+ to 4+ [3,4].

Examination of a Perls’ stain of a bone marrow film allows adequate assessment of erythroblast iron as long as a thinly spread area of the film is examined with optimal illumination. A proportion of normal erythroblasts have a few (one to five) fine iron-containing granules randomly distributed in...
the cytoplasm (Fig. 2.3). Such erythroblasts are designated sideroblasts. In haematologically normal subjects with adequate iron stores, 20–50% of bone marrow erythroblasts are sideroblasts [5–7]. Examination of an iron stain allows detection not only of an increased or decreased proportion of sideroblasts but also of abnormal sideroblasts. The latter include those in which siderotic granules are merely increased in size and number and those in which granules are also distributed abnormally within the cytoplasm, being sited in a ring around the nucleus rather than randomly (ring sideroblasts).

Table 2.1 Grading of bone marrow storage iron [1,2].

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No stainable iron</td>
</tr>
<tr>
<td>1+</td>
<td>Small iron particles just visible in reticulum cells using an oil objective</td>
</tr>
<tr>
<td>2+</td>
<td>Small, sparse iron particles in reticulum cells, visible at lower power</td>
</tr>
<tr>
<td>3+</td>
<td>Numerous small particles in reticulum cells</td>
</tr>
<tr>
<td>4+</td>
<td>Larger particles with a tendency to aggregate into clumps</td>
</tr>
<tr>
<td>5+</td>
<td>Dense, large clumps</td>
</tr>
<tr>
<td>6+</td>
<td>Very large clumps and extracellular iron</td>
</tr>
</tbody>
</table>

Fig. 2.1 Aspirate of normal BM: bluish-black iron in macrophages in a fragment. Perls’ stain ×377.

Fig. 2.2 Aspirate of normal BM: a fragment with no stainable iron. Perls’ stain ×377.
In certain pathological conditions, plasma cells contain haemosiderin inclusions which are irregular in shape and relatively large. On an MGG stain they are greenish-black (Fig. 2.4a). Their nature is confirmed by a Perls’ stain (Fig. 2.4b). Haemosiderin inclusions in plasma cells are observed mainly in iron overload (for example in haemochromatosis and transfusional siderosis) and in chronic alcoholism [8].

Problems and pitfalls
Since iron is distributed irregularly within bone marrow macrophages, it is necessary to assess a number of fragments before concluding that storage iron is absent or reduced. If necessary, a Perls’ stain can be performed on more than one bone marrow film. Stain deposit on the slide must be distinguished from haemosiderin. Careful examination will show that it is not related to cells and is often in another plane of focus.

Other cytochemical stains
Cytochemical stains are employed mainly in the investigation of acute leukaemia and the myelodysplastic syndromes (MDS). In acute leukaemia there may be numerous blast cells in the peripheral blood and it is then useful to perform cytochemical stains on blood and bone marrow in parallel. Cytochemical investigation of suspected MDS should be performed on bone marrow films since there are usually only small numbers of immature cells in the peripheral blood.

The techniques recommended for diagnosis and classification of acute leukaemia are either myeloperoxidase or Sudan black B staining, to identify cells showing granulocytic differentiation, plus a non-specific esterase or combined esterase stain, to identify cells showing monocytic differentiation. Enzyme cytochemistry for either α-naphthyl butyrate esterase or α-naphthyl acetate esterase is suitable as a ‘non-specific’ esterase staining method for the identification of monocytic differentiation. In a combined esterase stain either of these methods is combined with demonstration of naphthol AS-D chloroacetate esterase (chloro-acetate esterase), the latter to show granulocytic differentiation. The application of these stains will be discussed in Chapter 4.

Other cytochemical stains which are occasionally used include toluidine blue to demonstrate the metachromatic granules in basophils and mast cells and staining of cells of mast cell lineage for ε-aminocaproate. When immunophenotyping is available, periodic acid–Schiff (PAS) and acid phosphatase stains are redundant in the investigation of acute leukaemias, although differences can be observed between different types of acute lymphoblastic leukaemia (ALL) and acute myeloid leukaemia (AML) [9,10]. Either a Sudan black B or a myeloperoxidase stain should also be used in cases of suspected MDS to facilitate detection of Auer rods.
Cytochemistry now has little place in the investigation of lymphoproliferative disorders. However, the demonstration of tartrate-resistant acid phosphatase activity is still of value in the diagnosis of hairy cell leukaemia, particularly when a large panel of appropriate immunophenotyping reagents is not available.

**Histochemical stains on trephine biopsy sections**

**Perls’ stain for haemosiderin**

Because of the irregular distribution of iron within bone marrow macrophages, a biopsy may show the presence of iron when none has been detected in an aspirate. Haemosiderin can often be detected, particularly when it is increased, as golden brown refractile pigment in an unstained or H&E-stained section (Fig. 2.5). In a Giemsa-stained section it is greenish-blue (Fig. 2.6). An iron stain (Fig. 2.7) can be successfully carried out using either plastic-embedded or paraffin-embedded biopsy specimens. However, plastic-embedded specimens give more reliable results. Decalcification of a paraffin-embedded specimen, whether by acid decalcification or by chelation, leads to some leaching out of iron. Plastic-embedded samples are also superior for the detection of ring sideroblasts or other abnormal sideroblasts. These can sometimes also be detected.
Fig. 2.5 Section of BM showing haemosiderin within macrophages in an HIV-positive patient with iron overload. Paraffin-embedded, H&E ×376.

Fig. 2.6 Section of BM trephine biopsy specimen showing haemosiderin in stromal macrophages demonstrated by Giemsa staining. A distinctive yellow–green colour is obtained that is easily visible against background haemopoietic cell staining. Paraffin-embedded, Giemsa ×940.

Fig. 2.7 Section of normal BM: macrophage containing iron. Plastic-embedded, Perls’ stain ×940.
in paraffin-embedded bone marrow fragments but not in decalcified trephine biopsy sections. However, no technique for processing and staining of a biopsy specimen allows assessment of whether siderotic granules are normal or decreased; this requires an iron stain of an aspirate. Haemosiderin deposits in plasma cells may, however, be sufficiently large that they can be detected on sections of paraffin-embedded trephine biopsy specimens (Fig. 2.8).

Practice differs between laboratories as to whether a Perls’ stain for haemosiderin is performed routinely. If a bone marrow aspirate containing adequate fragments is available, then iron staining of trephine biopsy sections is redundant. However, it may well be more efficient for organizational reasons to perform an iron stain routinely rather than to perform it selectively only in those cases where it is likely to give information of specific diagnostic use.

Problems and pitfalls

The amount of iron that is leached out when a paraffin-embedded biopsy specimen is decalcified is variable and unpredictable. The amount of stainable iron is reduced and sometimes all stainable iron is removed. Loss of stainable iron is less with ethylene diamine tetra-acetic acid (EDTA) decalcification than with other methods. Because of the unpredictable leaching out of iron it is not possible to quantify iron accurately on a decalcified biopsy specimen. It is only possible to say that iron is present or increased but not that it is decreased or absent.

There are conflicting reports of the comparability of iron stains performed on aspirates and biopsy specimens, not all of which are readily explicable by the factors already mentioned. Lundin et al. [3] found that in 8% of cases iron was detectable in a biopsy specimen and not in an aspirate and in another 8% the reverse was true; by assessing other factors they were not able to establish that one or other method was more valid. Fong et al. [4] found that in 8% of patients iron was present in an aspirate but was not detectable in a biopsy sample; however, this was not due to the process of decalcification since it was noted with regard to sections of marrow fragments as well as for trephine biopsy sections. Conflicting findings were reported by Krause et al. [11] who found that iron was always detectable in a biopsy when it was present in an aspirate, but two thirds of patients with absent iron in an aspirate had detectable iron on a biopsy specimen. It is clear that minor variations in technique may be critical. Our own observations are that when specimens are decalcified using manual processing techniques there may be a failure to detect iron in a trephine biopsy specimen when it is clearly present in an aspirate. Iron stains performed on aspirates and biopsies should clearly be regarded as complementary.
Reticulin and collagen stains

Histological sections, either from particle preparations or trephine biopsy specimens, can be stained for reticulin using a silver-impregnation technique and also for collagen using a trichrome stain. We have found a Martius Scarlet Blue stain superior to a van Gieson stain for the identification of collagen. Reticulin and collagen deposition can be quantified as shown in Table 2.2 [12] and illustrated in Figs 2.9–2.13. The majority of haematologically normal subjects have a reticulin grade of 0 or 1 but occasional subjects have a grade of 2. There is a

Table 2.2 Quantification of bone marrow reticulin and collagen [12].

<table>
<thead>
<tr>
<th>Grade</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No reticulin fibres demonstrable</td>
</tr>
<tr>
<td>1</td>
<td>Occasional fine individual fibres and foci of a fine fibre network</td>
</tr>
<tr>
<td>2</td>
<td>Fine fibre network throughout most of the section; no coarse fibres</td>
</tr>
<tr>
<td>3</td>
<td>Diffuse fibre network with scattered thick coarse fibres but no mature collagen</td>
</tr>
<tr>
<td>4</td>
<td>Diffuse often coarse fibre network with areas of collagenization</td>
</tr>
</tbody>
</table>

Fig. 2.9 Section of normal BM: reticulin grade 0, showing no stainable fibres. Plastic-embedded, Gomori’s reticulin stain ×188.

Fig. 2.10 Section of normal BM: reticulin grade 1, showing scattered fine fibres. Plastic-embedded, Gomori’s reticulin stain ×188.
Fig. 2.11 Section of normal BM: reticulin grade 2, showing a fine fibre network but no coarse fibres. Paraffin-embedded, Gomori's reticulin stain ×195.

Fig. 2.12 Section of abnormal BM: reticulin grade 3, showing thick coarse fibres. Paraffin-embedded, Gordon and Sweet stain ×188.

Fig. 2.13 Section of abnormal BM: reticulin grade 4, showing a coarse fibre network; collagen was present. Paraffin-embedded, Gordon and Sweet stain ×188.
tendency for more reticulin to be detected in iliac crest biopsies than in sections of particles aspirated from the sternum. Reticulin is concentrated around blood vessels and close to bone trabeculae and these areas should be disregarded in grading reticulin deposition.

The term myelofibrosis is used to indicate deposition of collagen in the marrow and sometimes also to indicate increased reticulin deposition. To avoid any ambiguity it is preferable to either grade reticulin/collagen deposition as shown in Table 2.2 or to use the term ‘reticulin fibrosis’ for grade 3 fibrosis and ‘myelofibrosis’ for grade 4. The term myelosclerosis has also been used in various senses; it is best regarded as a synonym for myelofibrosis.

A reticulin stain should be performed on every trephine biopsy specimen. It has two major roles. Firstly, increased reticulin deposition provides non-specific evidence of an abnormality of the bone marrow. Secondly, focal abnormality in the pattern of reticulin deposition can be very useful in detecting abnormalities that might be overlooked in an H&E-stained section. Abnormal infiltrates may show an associated increase in reticulin deposition or, less often, there may be a general increase in reticulin deposition but with an absence of reticulin in an area that is heavily infiltrated by non-haemopoietic cells. Focal abnormalities that may be highlighted by a localized increase in reticulin deposition include granulomas and infiltrates of carcinoma or lymphoma cells.

Problems and pitfalls
To avoid confusion, pathologists should refer to reticulin and collagen deposition in a precise manner. Increased reticulin deposition provides evidence of a bone marrow abnormality but should not be over-interpreted since the causes are multiple. The causes of collagen deposition are fewer and this abnormality is therefore of more diagnostic significance. The significance of reticulin and collagen deposition is discussed in Chapter 3 (see page 130).

Other histochemical stains
Other potentially useful histochemical stains and their roles in diagnosis are shown in Table 2.3. A chloro-acetate esterase (Leder) stain is illustrated in Fig. 2.14.

Problems and pitfalls
The reactivity of histochemical stains is influenced by the choice of fixative, the method of embedding and the process of decalcification employed. Fixation in either Bouin’s or Zenker’s solution leads to reduced metachromatic staining of mast cells.

<table>
<thead>
<tr>
<th>Cytochemical stain</th>
<th>Role</th>
</tr>
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<tbody>
<tr>
<td>Chloro-acetate esterase (Leder)</td>
<td>Identification of granulocytic differentiation and mast cells</td>
</tr>
<tr>
<td>Periodic acid–Schiff (PAS)*</td>
<td>Staining of complex carbohydrates: identification of plasma cells</td>
</tr>
<tr>
<td></td>
<td>and megakaryocytes (staining is variable); identification of some</td>
</tr>
<tr>
<td></td>
<td>tumour cells; identification of fungi</td>
</tr>
<tr>
<td>Toluidine blue</td>
<td>Identification of mast cells</td>
</tr>
<tr>
<td>Alcian blue</td>
<td>Identification of cryptococci and some tumour cells; staining of</td>
</tr>
<tr>
<td></td>
<td>stromal mucins</td>
</tr>
<tr>
<td>Grocott’s methenamine silver stain (GMS)</td>
<td>Identification of fungi</td>
</tr>
<tr>
<td>Congo red stain</td>
<td>Identification of amyloid</td>
</tr>
<tr>
<td>Ziehl–Neelsen stain (ZN)</td>
<td>Identification of mycobacteria</td>
</tr>
<tr>
<td>Martius Scarlet Blue (MSB)</td>
<td>Staining of collagen and fibrin/fibrinoid</td>
</tr>
</tbody>
</table>

* Neutrophils are also PAS-positive.
with a Giemsa stain and also reduces or abolishes chloro-acetate esterase activity. Other histochemical stains are satisfactory with fixation in formalin or Bouin’s or Zenker’s solution. However, it should be noted that prolonged storage of formalin at high ambient temperatures can lead to formic acid production; if the formalin is unbuffered, inadvertent decalcification may occur during the process of fixation with resultant adverse effects on staining. Histochemical stains are satisfactory with both paraffin- and plastic-embedding. Acid decalcification impairs chloro-acetate esterase activity whereas EDTA decalcification does not. Over-exposure to EDTA reduces or abolishes Giemsa staining.

We have found that many of the proprietary combined fixation–decalcification solutions, which are sometimes used to achieve rapid processing, impair histochemical stains. For example, haematoxylin staining may be impaired so that nuclear detail and cytoplasmic basophilia are not apparent. Giemsa staining may be severely impaired.

**Immunophenotyping**

Antigens may be expressed on the surface of cells, within the cytoplasm or within the nucleus. Depending on the techniques applied for immunophenotyping, there may be detection of only surface membrane antigens or cytoplasmic and nuclear antigens may also be detected. Detection of antigens may be by means of polyclonal antibodies, raised in animals such as rabbits, but increasing use is being made of monoclonal antibodies produced by hybridoma technology. Many monoclonal antibodies, reactive with lymphoid or myeloid antigens, have been characterized at a series of international workshops and are described by cluster of differentiation (CD) numbers. A CD number refers to a group of antibodies that recognize the same antigen and also refers to the antigen expressed. It is important to note that monoclonal antibodies may recognize specific epitopes on antigens so that not all antibodies with the same CD number have exactly the same reactivity with normal and abnormal cells. A complete list of CD numbers is given in reference 13. Some important monoclonal antibodies have not yet been assigned a CD number, e.g. FMC7 and DBA44.

**Immunophenotyping by immunofluorescence flow cytometry**

If there are significant numbers of circulating abnormal cells, it is most convenient to perform flow cytometric immunophenotyping on a peripheral blood sample. Otherwise this procedure can be performed on a bone marrow aspirate or, alternatively, on a serous exudate, cerebrospinal fluid or a suspension of cells from a lymph node or other tissue. When peripheral blood is used, the procedure can be applied to either a mononuclear cell preparation or to whole blood in which the red cells have
been lysed [14]. The latter technique minimizes cell loss and potential artefacts that can be induced by exposure to Ficoll and density gradient separation. It also increases the speed and convenience of the procedure. Choice of appropriate proprietary lysis solutions is important to avoid reduction of expression of certain antigens [15].

The principle of flow cytometry is that cells bearing specific antigens are identified by means of a monoclonal antibody (or, occasionally, a polyclonal antiserum) labelled with a fluorochrome (Fig. 2.15). The flow cytometer permits classification of cells according to their light-scattering characteristics and the intensity of their fluorescence upon activation by laser light, detected after passing through an appropriate filter for the particular fluorochrome employed. Three or more fluorochromes can be used so that the simultaneous expression of two, three or more antigens can be studied. If permeabilization techniques are employed, cytoplasmic and nuclear antigens can be detected as well as those expressed on cell surfaces. Techniques are also available for the quantification of antigen expression.

Flow cytometry immunophenotyping is applicable to diagnosis and classification of haematological neoplasms. When expression of three or four antigens is assessed simultaneously it is also applicable to the detection of minimal residual disease.

Problems and pitfalls

Flow cytometry has the disadvantage that immunophenotype cannot be related directly to cytology. Results must always be interpreted in the light of the cytological features of the cells being studied.

When there are large numbers of circulating neoplastic cells, results of peripheral blood analysis are generally reliable. However, a low frequency of abnormal cells may not be detected. When flow cytometry is performed on cell suspensions from bone marrow or other tissues, results may be misleading in two circumstances. Firstly, an abnormal infiltrate may not be represented in the aspirate to any significant extent. This is often the case in follicular lymphoma with paratrabecular infiltration but may also occur in any lymphoma in which reticulin deposition is increased in the infiltrated area, interfering with aspiration of abnormal cells. Secondly, if neoplastic cells are outnumbered by reactive cells, as in Hodgkin’s disease and in T-cell-rich B-cell lymphoma, the immunophenotyping may relate only to reactive T lymphocytes, not to the minor population of neoplastic cells. In both of these circumstances immunohistochemistry is superior. In addition, correct techniques are of critical importance. For example, if gating techniques (see below) are used in order to determine the immunopheno-
type of a subpopulation of cells, it is essential to ensure that the gated cells are the neoplastic population.

Caution is required in interpreting flow cytometry findings during post-treatment follow-up of ALL. Normal immature lymphoid cells, known as haematogones, express CD10, CD34 and terminal deoxynucleotidyl transferase (TdT) and can thus be confused with residual leukaemic cells [16] unless the strength of expression of antigens is also considered. The detection of persisting cells with an aberrant combination of antigens is more reliable, as is polymerase chain reaction (PCR) analysis for rearranged immunoglobulin heavy chain (IGH) or T-cell receptor (TCR) genes (see below).

Immunocytochemistry

By convention, the term immunocytochemistry refers to the study of the antigen expression of cells by means of polyclonal or monoclonal antibodies applied to fixed cells on glass slides. The material investigated may be either a blood or bone marrow film or a cytocentrifuged preparation of washed mononuclear cells isolated from blood or bone marrow. The reaction of antibodies with cells carrying a specific antigen is detected by either: (i) direct labelling of the primary antibody with an enzyme such as peroxidase or alkaline phosphatase; or (ii) an indirect method using a second, labelled antibody that recognizes the first (e.g. a secondary antibody reactive with mouse immunoglobulins when the primary antibody is murine). A variety of indirect methods are available. Indirect labelling techniques offer the advantage of increased sensitivity but are more time-consuming to perform than direct labelling.

The use of washed, separated cells in cytocentrifuge preparations is necessary for immunocytochemistry to detect surface membrane immunoglobulins, including κ and λ light chains. Plasma immunoglobulins interfere with the staining if blood films or films of bone marrow aspirates are used. For detection of most other antigens, either cytocentrifuge preparations or wedge-spread films are satisfactory. If there are significant numbers of abnormal cells in the circulating blood, then a peripheral blood sample is very satisfactory for immunocytochemistry. Otherwise study of a bone marrow aspirate is necessary.

Immunocytochemistry can also be used to demonstrate the product of an oncogene or a cancer-suppressing gene. For example, PML protein, the product of the gene that is rearranged and dysregulated in acute promyelocytic leukaemia, can be demonstrated with a fluorochrome-labelled monoclonal antibody and an abnormal pattern of distribution can be shown in this type of leukaemia. Similarly, a labelled antibody can be used to demonstrate increased expression of p53 protein when the gene is dysregulated; p53 protein is below the level of detection when only the wild type gene is present.

Problems and pitfalls

Immunocytochemistry has the advantage that reactivity with an antibody can be related to cell morphology. However, it should be noted that cytocentrifugation introduces artefactual changes, such as nuclear lobulation. It is useful to examine an MGG-stained cytocentrifuge preparation in parallel with the immunocytochemical stains and wedge-spread films.

Cytochemistry is slow and labour intensive and thus is not suitable for large workloads or for producing rapid results. Interpretation is subjective and, because only a small number of cells can be assessed, results are imprecise. Some useful antibodies, for example FMC7, cannot be used successfully for immunocytochemistry although they are very reliable with flow cytometry.

Relative advantages of flow cytometry and immunocytochemistry

Flow cytometry has various advantages over immunocytochemistry and is therefore the preferred technique where it is available as:

1 it is rapid and less labour intensive;
2 it permits large numbers of cells to be analysed so that the percentage of cells bearing a specific antigen is estimated much more precisely and minor populations of cells may be identified;
3 multiple directly labelled antibodies can be used to study the co-expression of two, three or even four antigens;
4 it is possible to ‘gate’ for cells having particular characteristics in order to investigate antigen expression by a defined population (‘gating’ or selection of
a subpopulation may be based on light-scattering characteristics of cells or on expression of a specific antigen); and
5 the amount of antigen expressed on a specific population of cells can be quantified, whereas immunocytochemistry is not quantitative.

These advantages of flow cytometry mean that it can be used to identify minor normal populations, such as CD34-positive haemopoietic stem cells, and minor abnormal populations showing atypical combinations of antigen expression, as in the detection of minimal residual disease in patients treated for haematological neoplasms.

There are two important disadvantages of flow cytometry in comparison with immunocytochemistry: 1 without modification, the technique detects only surface membrane antigens and not antigens expressed within the cytoplasm or in the nucleus; and 2 cytological features of the cells studied cannot be appreciated.

The first disadvantage can be overcome readily by the use of techniques for permeabilizing cells so that antigens expressed in the nucleus or in the cytoplasm can be detected. The second defect cannot be overcome easily but use of light-scattering characteristics of cells does at least permit a determination of whether specific antigens are expressed on large or small cells. Results of flow cytometry should not be interpreted in isolation; the cytological features of the cells being studied must be taken into account.

Immunocytochemistry has the advantage over flow cytometry that the precise cytological features of cells bearing a certain antigen can be recognized. As mentioned above, however, it is very labour intensive and more time-consuming than flow cytometry and results are not quantitative. Double or multiple antigen combinations on individual cells cannot be demonstrated routinely. Although techniques exist for sequential immunocytochemical staining of several antigens in the same preparation, these are largely restricted to use as research tools at present because of their practical difficulties.

Antibodies for flow cytometry and immunocytochemistry

Antibodies to be used in flow cytometry are selected depending upon the purpose of the investigation. There are relatively few circumstances in which a single antibody is used in isolation. Specific combinations are used for investigation of suspected acute leukaemia or possible lymphoproliferative disorders. Suggested panels are provided in Tables 2.4 and 2.5.

Table 2.4 Monoclonal and other antibodies useful in immunocytochemistry and flow cytometry immunophenotyping in suspected acute leukaemia.

<table>
<thead>
<tr>
<th>Primary panel</th>
<th>CD117, CD13*, CD33, anti-MPO, CD65</th>
</tr>
</thead>
<tbody>
<tr>
<td>For detection of myeloid differentiation</td>
<td>CD19, CD22*, CD79a</td>
</tr>
<tr>
<td>For detection of B-lymphoid differentiation</td>
<td>CD2, CD3*, anti-TCRαβ, anti-TCRγδ</td>
</tr>
<tr>
<td>For detection of T-lymphoid differentiation</td>
<td>Anti-TdT (anti-terminal deoxynucleotidyl transferase), CD34, HLA-DR</td>
</tr>
<tr>
<td>For detection of immature cells</td>
<td></td>
</tr>
<tr>
<td>Secondary panel</td>
<td></td>
</tr>
<tr>
<td>For further investigation of myeloid differentiation</td>
<td>Anti-glycophorin for erythroid differentiation, CD41 (or CD61) for megakaryocyte differentiation, CD14 for monocytic differentiation, CD11b for granulocytic or monocytic maturation</td>
</tr>
<tr>
<td>For further investigation of B-lineage differentiation</td>
<td>CD10, cytoplasmic immunoglobulin, surface membrane immunoglobulin</td>
</tr>
<tr>
<td>For further investigation of T-lineage differentiation</td>
<td>CD1a, CD4, CD5, CD8</td>
</tr>
</tbody>
</table>

* Testing is more sensitive if cytoplasmic rather than surface membrane antigen is tested for, either by ‘permeabilizing’ cells or by using immunocytochemistry rather than flow cytometry.
In addition, flow cytometry can be used for:
1 quantification of CD34-positive haemopoietic stem cells for stem cell harvesting and transplantation;
2 quantification of CD4-positive lymphocytes for assessment of immune status in HIV infection; and
3 quantification of DNA for cell cycle and ploidy studies; e.g. for detection of hyperdiploidy in ALL.

Problems and pitfalls
Because many immunophenotypic markers are not lineage-specific it is always necessary to use a panel of antibodies rather than relying on reactivity with a single antibody. Immunophenotypic markers showing good lineage specificity include CD79a and CD79b for the B lineage, CD3 for the T lineage and myeloperoxidase for myeloid cells. However, it should be noted that cross-reactivity of CD79a with some cases of T-lineage ALL has been reported [17].

Immunophenotypic markers with poor lineage specificity include TdT, HLA-DR, CD7 and CD10.

**Table 2.5** Monoclonal and other antibodies useful in immunophenotyping suspected chronic lymphoproliferative disorders.

<table>
<thead>
<tr>
<th><strong>Primary panel</strong></th>
<th><strong>Secondary panel</strong></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>To establish lineage</strong></td>
<td><strong>To establish clonality of B-lineage lymphoproliferative disorders and to establish the strength of expression of surface membrane immunoglobulin</strong></td>
</tr>
<tr>
<td>A pan-B marker such as CD79a, CD19 or CD24 and a pan-T marker such as CD2 or CD3</td>
<td>Anti-κ and anti-λ.</td>
</tr>
<tr>
<td><strong>To distinguish B-lineage chronic lymphocytic leukaemia from other less common B- and T-lineage disorders</strong></td>
<td>CD79b (or CD22), CD5, CD23 and FMC7 (CLL cells are usually CD5 and CD23 positive, CD22, CD79b and FMC7 weak or negative and show weak expression of SmIg; the reverse pattern is usual in most types of B-lineage NHL)</td>
</tr>
<tr>
<td><strong>For further investigation of suspected B-lineage lymphoproliferative disorder</strong></td>
<td>CD10 (more often positive in follicular lymphoma); CD11c, CD25, CD103 and HC2 (for suspected hairy cell leukaemia); anti-cyclin D1 (suspected mantle cell lymphoma); CD38, CD79a, CD138 and CyIg (for suspected plasma cell or lymphoplasmacytoid neoplasm)</td>
</tr>
<tr>
<td><strong>For further investigation of suspected T-lineage lymphoproliferative disorder</strong></td>
<td>CD4, CD8 (usually positive in large granular lymphocyte leukaemia), CD7 (usually positive in T-PLL), CD25 (usually positive in ATLL); CD11b, CD16, CD56, CD57 (for suspected large granular lymphocyte or NK cell leukaemia/lymphoma)</td>
</tr>
<tr>
<td><strong>Anti-terminal deoxynucleotidyl transferase (anti-TdT)</strong></td>
<td>Recommended if it is not certain if cells are precursor lymphocytes (ALL or lymphoblastic lymphoma) or mature lymphocytes</td>
</tr>
<tr>
<td><strong>For planning of therapy</strong></td>
<td>CD20 (or any other antigen that may be a target of monoclonal antibody therapy)</td>
</tr>
</tbody>
</table>

**Immunohistochemistry**
Immunohistochemistry refers to the demonstration of antigens in histological tissue sections. It has advantages and disadvantages in relation to flow cytometry and immunocytochemistry. For practical purposes, flow cytometry and immunohistochemistry should be regarded as complementary investigations.

In flow cytometry, a wider range of antibodies can be used and quantification of antigen expression can be achieved. There is also less likelihood of non-specific staining in flow cytometry than in immunohistochemistry. However, immunohistochemistry has the advantage that immunophenotypic information can be obtained and assessed in
association with preserved information regarding the spatial organization of labelled and unlabelled cells. Immunohistochemistry also permits assessment of the cytological features of cells expressing particular antigens, in a context which is often more familiar than that offered by immunocytochemistry (particularly cytocentrifuge preparations, in which significant morphological artefacts are induced by the centrifugation technique itself).

Because a large number of antigens can be stained individually in adjacent thin tissue sections, the lack of easy techniques for double-staining is not a major problem in immunohistochemistry performed for diagnostic and staging purposes. In a complex infiltrate it is relatively easy to demonstrate a variety of cell types differing in morphological and immunophenotypic characteristics. For example, in T-cell-rich B-cell lymphoma, the large, neoplastic cells can be shown to have a B-cell phenotype while the more numerous T cells are seen to be cytologically normal small lymphocytes.

Immunohistochemistry can permit the identification and characterization of an abnormal bone marrow infiltrate not represented in the patient’s aspirate sample. This situation usually arises when there is significant reticulin fibrosis associated with an infiltrate, which hinders aspiration of cells from the involved area or areas of bone marrow. Such reticulin fibrosis occurs frequently in follicular lymphoma and almost invariably accompanies bone marrow infiltration by Hodgkin’s disease.

When immunohistochemistry first gained widespread use in histopathology during the 1970s, it was commonly assumed that decalcification, particularly with methods involving exposure to acids, led to destruction of many antigens. This has proved to be untrue and the procedure can be performed very successfully with acid- or EDTA-decalcified trephine biopsies as well as with non-decalcified samples embedded in methacrylate resins. A few important technical modifications were required to overcome the different performance of bone marrow trephine biopsy specimens relative to other formalin-fixed tissues. For example, early antigen retrieval techniques, involving tissue digestion by proteolytic enzymes to reverse protein–protein binding induced by formalin fixation, were responsible for many initial poor results of immunohistochemistry in bone marrow trephine biopsy specimens as a consequence of degradation of the antigenic target. Prior exposure to acid appears to render formalin-fixed tissue more susceptible to proteolysis and leads to degradation of some antigenic targets during incubation with the enzyme. In general, considerably shortened incubation times have therefore been found to be beneficial with these antigen retrieval methods. The major advance, however, has come through development of wet-heat methods for antigen retrieval, exposing tissue sections to acid or alkaline solutions in combination with microwave oven or pressure cooker heating. This has been as important for immunohistochemistry as a general tool in histopathology as it has been for bone marrow trephine biopsy specimens. It has encouraged a huge expansion in the development of new monoclonal antibodies for diagnosis in addition to making possible excellent results using many existing antibodies that were previously unsuccessful.

The second technical modification required for successful immunohistochemistry in bone marrow biopsy specimens was necessary to minimize non-specific staining due to endogenous enzyme activity. Most methods employ indirect labelling techniques with either peroxidase or alkaline phosphatase conjugated to the secondary antibody. The enzyme generates an insoluble, coloured product from a chromogenic substrate to permit visualization of the primary antigen–antibody interaction. Cells of the granulocyte series, particularly eosinophils, are rich in endogenous peroxidase activity and bone marrow stroma contains dendritic cells that are rich in alkaline phosphatases. When performing immunohistochemistry on bone marrow trephine biopsy specimens, additional steps are required to block such endogenous enzyme activities and minimize non-specific staining. To compound this problem, highly efficient amplification steps are included in many current immunohistochemistry methods, increasing sensitivity by exploiting the extremely high binding affinity of avidin or streptavidin for biotin. Endogenous biotin activity, particularly in mast cells, may also therefore require specific blockade to avoid false-positive staining.

In practice, the necessary technical modifications are easy to incorporate to achieve excellent results in bone marrow trephine biopsy samples with a range of primary antibodies. While this range is somewhat limited compared with that used in flow cytometry,
it is nonetheless extensive. Moreover, since much bone marrow trephine biopsy immunohistochemistry is performed in the investigation of lymphoproliferative disorders, it is valuable that the antibodies which can be used successfully are also entirely suitable for use with other formalin-fixed tissue samples such as biopsied lymph nodes.

Immunohistochemistry can be used to demonstrate surface membrane, cytoplasmic and nuclear antigens. It can be used specifically to provide molecular genetic information, e.g. by demonstration of the protein product of oncogenes, such as ALK, BCL1 and BCL2, or of cancer-suppressing genes, such as p53.

Tables 2.6 and 2.7 give details of useful antibodies for the immunohistochemical detection of antigen expression on haemopoietic and lymphoid cells in bone marrow trephine biopsies.

Table 2.6 Antigens expressed by myeloid cells and demonstrable by immunohistochemistry in fixed, decalcified bone marrow trephine biopsy specimens.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD34</td>
<td>QBEnd 10</td>
<td>Primitive haemopoietic cells</td>
<td>Endothelial cells also positive</td>
</tr>
<tr>
<td>CD45</td>
<td>PD7/26, RP2/18, RP2/22</td>
<td>Lymphoid, granulocytic and monocytic cells</td>
<td>Proteolytic pre-treatment abolishes granulocytic and monocytic reactivity</td>
</tr>
<tr>
<td>Lysozyme (muramidase)</td>
<td>Polyclonal antisera</td>
<td>Granulocytic and monocytic cells</td>
<td></td>
</tr>
<tr>
<td>Myeloperoxidase</td>
<td>Polyclonal antisera</td>
<td>Granulocytic and monocytic cells</td>
<td></td>
</tr>
<tr>
<td>Alpha-1-antitrypsin</td>
<td>Polyclonal antisera</td>
<td>Granulocytic and monocytic cells</td>
<td></td>
</tr>
<tr>
<td>CD66e</td>
<td>85A12</td>
<td>Granulocytic cells</td>
<td>Many metastases of epithelial origin also positive</td>
</tr>
<tr>
<td>CD68—broad specificity</td>
<td>KP1</td>
<td>Granulocytic and monocytic cells, including osteoclasts</td>
<td>Mast cells also positive</td>
</tr>
<tr>
<td>CD68—monocyte restricted</td>
<td>PG-M1</td>
<td>Monocytic cells, including osteoclasts</td>
<td>Mast cells also positive</td>
</tr>
<tr>
<td>CD163</td>
<td>10D6</td>
<td>Monocyte lineage cells including osteoclasts</td>
<td></td>
</tr>
<tr>
<td>CD14</td>
<td>NCL-CD14-223</td>
<td>Monocytic cells</td>
<td>Developing monocytes strongly positive; macrophages weak or negative</td>
</tr>
<tr>
<td>Neutrophil elastase</td>
<td>NP57</td>
<td>Granulocytic cells</td>
<td>Promyelocytes and myelocytes strongly positive; metamyelocytes and mature neutrophils stain weakly or are negative</td>
</tr>
<tr>
<td>CD15</td>
<td>LeuM1, BY87</td>
<td>Monocytic and granulocytic cells, especially late granulocyte precursors</td>
<td>Membrane and cytoplasmic staining but membranes negative after proteolytic pre-treatment; expressed by neoplastic cells of Hodgkin’s disease</td>
</tr>
</tbody>
</table>
### Table 2.6 (cont’d)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calprotectin (previously called calgranulin)</td>
<td>Mac387</td>
<td>Late granulocyte precursors and monocytic cells</td>
<td>Macrophages weakly stained or negative</td>
</tr>
<tr>
<td>Eosinophil major basic protein</td>
<td>BMK13</td>
<td>Eosinophil granulocytes</td>
<td></td>
</tr>
<tr>
<td>Mast cell tryptase</td>
<td>AA1</td>
<td>Mast cells</td>
<td></td>
</tr>
<tr>
<td>CD117</td>
<td>57ASD8</td>
<td>Mast cells</td>
<td>Avoidance of problems with diffusion of reaction product and non-specific background staining needs careful pre-treatment</td>
</tr>
<tr>
<td>CD1a</td>
<td>MTB1, JPM30</td>
<td>Langerhans cells</td>
<td>Few, if any, such cells are normally present; used in diagnosis of Langerhans cell histiocytosis</td>
</tr>
<tr>
<td>Glycophorin A (α-sialoglycoprotein)</td>
<td>JC159, BRIC101</td>
<td>Erythroid cells</td>
<td>Very early proerythroblasts not stained</td>
</tr>
<tr>
<td>Glycophorin C (β-sialoglycoprotein)</td>
<td>Ret40f</td>
<td>Erythroid cells</td>
<td>Expression seen in earlier proerythroblasts than α-sialoglycoprotein; cross-reactivity with myeloblasts is seen in some cases of AML</td>
</tr>
<tr>
<td>Spectrin</td>
<td>Polyclonal antisera</td>
<td>Erythroid cells</td>
<td>No commercially available antibody at present [18]</td>
</tr>
<tr>
<td>Haemoglobin A</td>
<td>Polyclonal antisera</td>
<td>Haemoglobinized erythroid cells</td>
<td>Early erythroid precursors weak or negative</td>
</tr>
<tr>
<td>An epitope on the ABO blood group H glycoprotein</td>
<td>BNH9</td>
<td>Erythroid cells</td>
<td>Expressed from earliest recognizable stages of erythroid differentiation; also expressed by endothelial cells, megakaryocytes and cells of some large cell lymphomas</td>
</tr>
<tr>
<td>CD61</td>
<td>Y2/51</td>
<td>Megakaryocytes</td>
<td>Variable staining between cells; many early or dysplastic megakaryocytes unstained</td>
</tr>
<tr>
<td>CD42b</td>
<td>MM2/174</td>
<td>Megakaryocytes</td>
<td>Strong, uniform cytoplasmic staining, including early and dysplastic forms</td>
</tr>
<tr>
<td>Von Willebrand factor (previously known as Factor VIII-related antigen)</td>
<td>F8/86 and various polyclonal antisera</td>
<td>Megakaryocytes</td>
<td>Strong cytoplasmic staining; variable results with early and dysplastic forms</td>
</tr>
</tbody>
</table>
Table 2.7 Antigens expressed by lymphoid cells (B, T and NK lineages) and demonstrable by immunohistochemistry in fixed, decalcified bone marrow trephine biopsy specimens.

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD45</td>
<td>PD7/26, RP218, RP2/22</td>
<td>Lymphoid, granulocytic and monocyctic cells</td>
<td>Proteolytic pre-treatment minimizes granulocytic and monocyctic staining</td>
</tr>
<tr>
<td>Terminal deoxynucleotidyl transferase (TdT)</td>
<td>NPT26</td>
<td>Primitive lymphoid cells and some primitive myeloid cells</td>
<td>In addition to ALL, up to 15% of AML are positive</td>
</tr>
<tr>
<td>CD10</td>
<td>56C6</td>
<td>Subsets of B cells</td>
<td>Expresses in common and pre-B ALL, Burkitt’s lymphoma and follicle centre cell lymphoma; also expressed by a subset of marrow stromal cells</td>
</tr>
<tr>
<td>CD20</td>
<td>L26</td>
<td>Most B cells</td>
<td>Some early B-lineage lymphoid cells and cells showing plasmacytic differentiation are negative</td>
</tr>
<tr>
<td>CD79a</td>
<td>Mb1, HM47/A9, JCB117</td>
<td>Most B cells</td>
<td>Includes early B-lineage lymphoid cells and those with plasmacytic differentiation; may be negative in non-secretory myeloma; megakaryocytes are weakly positive</td>
</tr>
<tr>
<td>CDw75</td>
<td>LN1</td>
<td>B cells</td>
<td>Preferential staining of large, transformed B cells (e.g. centroblasts and immunoblasts)</td>
</tr>
<tr>
<td>CD45RA</td>
<td>4KB5</td>
<td>B cells and a subset of T-lineage lymphoid cells</td>
<td>Small, mature B cells stain preferentially</td>
</tr>
<tr>
<td>Immunoglobulin light chains—κ and λ</td>
<td>Polyclonal antisera are usually used</td>
<td>Plasma cells</td>
<td>Expression by other B cells usually too weak to be detected; excessive background staining may occur due to plasma immunoglobulins within the tissue</td>
</tr>
<tr>
<td>Immunoglobulin heavy chains—γ, α, μ, ε, δ</td>
<td>Polyclonal antisera are usually used</td>
<td>Plasma cells</td>
<td>Expression by other B cells usually too weak to be detected; excessive background staining may occur due to plasma immunoglobulins within the tissue</td>
</tr>
<tr>
<td>Rough endoplasmic reticulum-associated antigen (p63)</td>
<td>VS38c</td>
<td>Plasma cells</td>
<td>Osteoblasts and a subset of stromal cells also stain</td>
</tr>
<tr>
<td>CD138 (syndecan)</td>
<td>B-B4</td>
<td>Plasma cells</td>
<td>Some carcinomas and large cell NHL are positive</td>
</tr>
<tr>
<td>CD38</td>
<td>AT13/5</td>
<td>Plasma cells</td>
<td>Also expressed by thymocytes, early B cells, germinal centre B cells and some erythroid cells and neutrophils</td>
</tr>
<tr>
<td>Unclustered</td>
<td>DBA44</td>
<td>B cells</td>
<td>Relative specificity for hairy cell leukaemia; red cell membranes and some macrophages may also stain</td>
</tr>
<tr>
<td>Tartrate-resistant acid phosphatase</td>
<td>9CS and 14G6</td>
<td>Hairy cell leukaemia</td>
<td>Mast cells, Langerhans cells, macrophages and osteoclasts also stain [19]</td>
</tr>
<tr>
<td>CD23</td>
<td>1B12</td>
<td>Subset of B cells</td>
<td>Small lymphocytic lymphoma/CLL positive; mantle cell, lymphoplasmacytoid and follicle centre cell lymphomas negative</td>
</tr>
</tbody>
</table>
SPECIAL TECHNIQUES

CD5 4C7 Subset of B cells, most T cells
Small lymphocytic lymphoma/CLL and mantle cell lymphoma positive; lymphoplasmacytoid and follicle centre cell lymphomas negative

BCL2 Bcl-100/D5, I24 T cells, mantle zone B lymphocytes and follicle centre cell lymphoma
Non-neoplastic germinal centre cells are negative; other B-cell lymphomas and some haemopoietic cells are positive; it is difficult to interpret positive staining in the bone marrow

Cyclin D1 DCS-6, P2D11F11 Mantle cell lymphoma Apoptotic nuclei and some endothelial cell nuclei are positive; immunostaining is technically difficult

CD2 AB75 Most T cells Also expressed by some monocytes

CD3 CD3-12 and polyclonal antisera Most T cells More specific than CD2; the monoclonal antibody is superior in performance to polyclonal antisera

T-cell receptor β chain βF1 T cells expressing αβ T-cell receptor The majority of CD3-positive T cells and cells of many T-lineage neoplasms

CD45RO—broad specificity UCHL-1 Antigen-experienced T cells (membrane expression) Granulocytes, monocytes and macrophages are also positive (cytoplasmic expression); excessive decalcification may lead to non-specific nuclear staining

CD45RO—T-cell restricted OPD4 Antigen-experienced T cells (membrane expression) Less reactivity with non-lymphoid lineage cells than with UCHL1

CD43 MT1, DFT1 Most T cells and a subset of B cells; lymphocytic lymphoma/CLL and mantle cell lymphoma are usually positive; follicular lymphoma, lymphoplasmacytic lymphoma and marginal zone lymphomas are negative

CD4 1F6 T-cell subset There is often poor localization of reaction product to individual cells; macrophages are also positive

CD8 4B11 T-cell subset

CD56 1B6 Natural killer cells and some cytotoxic T cells Nerve fibres, neuro-ectodermal tumours, some leukaemic myeloblasts and cells of some cases of multiple myeloma are positive

CD57 Leu7, NC1 Natural killer cells and some cytotoxic T cells Nerve fibres and neuro-ectodermal tumours are positive

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Table 2.7 (cont’d)

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD5</td>
<td>4C7</td>
<td>Subset of B cells, most T cells</td>
<td>Small lymphocytic lymphoma/CLL and mantle cell lymphoma positive; lymphoplasmacytoid and follicle centre cell lymphomas negative</td>
</tr>
<tr>
<td>BCL2</td>
<td>Bcl-100/D5, I24</td>
<td>T cells, mantle zone B lymphocytes and follicle centre cell lymphoma</td>
<td>Non-neoplastic germinal centre cells are negative; other B-cell lymphomas and some haemopoietic cells are positive; it is difficult to interpret positive staining in the bone marrow</td>
</tr>
<tr>
<td>Cyclin D1</td>
<td>DCS-6, P2D11F11</td>
<td>Mantle cell lymphoma (nuclear expression)</td>
<td>Apoptotic nuclei and some endothelial cell nuclei are positive; immunostaining is technically difficult</td>
</tr>
<tr>
<td>CD2</td>
<td>AB75</td>
<td>Most T cells</td>
<td>Also expressed by some monocytes</td>
</tr>
<tr>
<td>CD3</td>
<td>CD3-12 and polyclonal antisera</td>
<td>Most T cells</td>
<td>More specific than CD2; the monoclonal antibody is superior in performance to polyclonal antisera</td>
</tr>
<tr>
<td>T-cell receptor β chain</td>
<td>βF1</td>
<td>T cells expressing αβ T-cell receptor</td>
<td>The majority of CD3-positive T cells and cells of many T-lineage neoplasms</td>
</tr>
<tr>
<td>CD45RO—broad specificity</td>
<td>UCHL-1</td>
<td>Antigen-experienced T cells (membrane expression)</td>
<td>Granulocytes, monocytes and macrophages are also positive (cytoplasmic expression); excessive decalcification may lead to non-specific nuclear staining</td>
</tr>
<tr>
<td>CD45RO—T-cell restricted</td>
<td>OPD4</td>
<td>Antigen-experienced T cells (membrane expression)</td>
<td>Less reactivity with non-lymphoid lineage cells than with UCHL1</td>
</tr>
<tr>
<td>CD43</td>
<td>MT1, DFT1</td>
<td>Most T cells and a subset of B cells; lymphocytic lymphoma/CLL and mantle cell lymphoma are usually positive; follicular lymphoma, lymphoplasmacytic lymphoma and marginal zone lymphomas are negative</td>
<td></td>
</tr>
<tr>
<td>CD4</td>
<td>1F6</td>
<td>T-cell subset</td>
<td>There is often poor localization of reaction product to individual cells; macrophages are also positive</td>
</tr>
<tr>
<td>CD8</td>
<td>4B11</td>
<td>T-cell subset</td>
<td>Nerve fibres, neuro-ectodermal tumours, some leukaemic myeloblasts and cells of some cases of multiple myeloma are positive</td>
</tr>
<tr>
<td>CD56</td>
<td>1B6</td>
<td>Natural killer cells and some cytotoxic T cells</td>
<td>Nerve fibres and neuro-ectodermal tumours are positive</td>
</tr>
<tr>
<td>CD57</td>
<td>Leu7, NC1</td>
<td>Natural killer cells and some cytotoxic T cells</td>
<td>Nerve fibres and neuro-ectodermal tumours are positive</td>
</tr>
</tbody>
</table>
Immunohistochemical stains are illustrated in Figs 2.16–2.19. Monoclonal antibodies useful in the detection of micro-organisms are shown in Table 3.1 and those for use in the diagnosis of non-haemopoietic malignancy in Table 10.1.

**Table 2.7 (cont’d)**

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Antibody</th>
<th>Specificity</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD30</td>
<td>BerH2</td>
<td>Reed–Sternberg cells and mononuclear Hodgkin’s cells, cells of anaplastic large cell lymphoma and some other pleomorphic large cell lymphomas</td>
<td>Plasma cells and some erythroid precursors positive if proteolytic pre-treatment is used; wet-heat antigen retrieval is preferred; some carcinomas, especially embryonal, are positive</td>
</tr>
<tr>
<td>CD15</td>
<td>LeuM1, BY87</td>
<td>Neoplastic cells in classical Hodgkin’s disease (but may be negative in up to 15% of cases of Hodgkin’s disease including lymphocyte-predominant)</td>
<td>Granulocytic and monocytic cells positive, particularly late granulocyte precursors</td>
</tr>
<tr>
<td>Epithelial membrane antigen</td>
<td>GP1.4</td>
<td>Cells of anaplastic large cell lymphoma</td>
<td>Some plasma cells are positive; neoplastic cells in some cases of Hodgkin’s disease and many carcinomas are positive; some cases of anaplastic large cell lymphoma are negative</td>
</tr>
<tr>
<td>Anaplastic lymphoma-associated kinase</td>
<td>ALK-1</td>
<td>Cells of anaplastic large cell lymphoma</td>
<td>More likely to be positive in childhood than adult cases</td>
</tr>
<tr>
<td>Ki67 antigen</td>
<td>Ki67, MM1, M1B-1</td>
<td>Proliferating cells (nuclear expression)</td>
<td>Useful for assessing grade of lymphoma from infiltrates in bone marrow; proliferating haemopoietic cells are also positive</td>
</tr>
</tbody>
</table>

**Fig. 2.16** Section of trephine biopsy specimen from a patient with a myelodysplastic syndrome showing elastase-positive granulocyte precursors. Paraffin-embedded, immunoperoxidase technique with anti-elastase McAb NP5 ×960.

Problems and pitfalls

**Antigen cross-reactivity between cell types.** Antigen cross-reactivity between different types of cell is a significant problem in immunohistochemistry...
Fig. 2.17 Section of trephine biopsy specimen from a patient with M6 AML showing erythroblasts, one of which is binucleated. Paraffin-embedded, immunoperoxidase technique with antiglycophorin antibody McAb Ret40f ×940.

Fig. 2.18 Section of trephine biopsy specimen from a patient with a myelodysplastic syndrome showing a cluster of megakaryocytes. Paraffin-embedded, immunoperoxidase technique with CD61 McAb ×940.

Fig. 2.19 Section of trephine biopsy specimen showing pericapillary plasma cells. Paraffin-embedded, immunoperoxidase technique with McAb VS38c ×940.
There is a tendency to regard antigen expression as being lineage-specific, whereas expression is more accurately considered as lineage-associated or lineage-restricted. Many antigens familiar in the context of expression by T cells, B cells or both in lymphoid tissues are also expressed by myeloid lineages. This is particularly the case with T-cell-associated antigens, which are widely expressed by cells of granulocytic and monocytic lineages. CD43 should be avoided as a T-cell marker in bone marrow because of extensive cross-reactivity of this type. Antibodies reactive with CD45RO should be selected with care; the clone OPD4 stains T cells preferentially, with little granulocytic and monocytic reactivity, while UCHL-1 often stains cells of the latter types strongly. Expression of CD45RO by granulocytes and monocytes can be distinguished from that shown by T cells since the former is cytoplasmic, whereas T cells show membrane staining. Bone marrow stromal macrophages express CD4 and, because of the poor localization of a rather weak signal that is often seen with the currently available antibody, accurate assessment of CD4-positive T cells may not be possible. Monoclonal antibodies reactive with CD3 are currently the best and most specific T-cell markers for use with bone marrow trephine biopsy sections.

For B cells, CD79a is probably the best antigenic target but some antibody clones cross-react with vascular smooth muscle and megakaryocytes; these reactivities rarely cause problems in interpretation. In our experience, many bone marrow B cells appear negative or react only weakly for CD20; both CD79a and an alternative B-cell marker, 4KB5 (CD45RA), show consistently more small B cells than does CD20 both in normal bone marrow and in low grade lymphoproliferative disorders. For this reason, trephine biopsy immunostaining for assessment of CD20 expression for therapeutic purposes should not, at present, be regarded as reliable if a negative result is obtained. It should be noted that L26, the CD20 monoclonal antibody used to detect reactivity in trephine biopsy specimens, detects an intracellular epitope, whereas the monoclonal antibody used for therapy reacts with a surface membrane epitope. Antibodies directed against CD5 and BCL2 react with T cells as well as subsets of normal and neoplastic B cells; results should be interpreted with care in bone marrow lymphoid infiltrates, in which non-neoplastic T cells frequently predominate.

The specificity of CD45 antibodies for lymphoid cells can be improved if myeloid reactivity is reduced by proteolysis (performed as for antigen retrieval). Antibodies reactive with CD30 may cross-react with plasma cells and, depending on fixation, with erythroid cells. Expression in these cell types is cytoplasmic rather than Golgi- or membrane-associated as in Reed–Sternberg cells or cells of anaplastic large cell lymphoma. This cross-reactivity is unlikely to cause difficulty in the interpretation of possibly lymphoid infiltrates. However, it can be abolished by use of wet heat for antigen retrieval, rather than proteolysis.
Endothelial cell expression of CD34 can mimic reactivity in haemopoietic cells if tiny capillaries are viewed in cross-section. However, the granular nature of haemopoietic cell CD34 expression can be distinguished from the homogeneous pattern seen in endothelium.

*Problems relating to fixation and decalcification.* Use of proprietary combined fixative–decalcifier solutions can lead to extensive loss of immunoreactivity within tissues, as can excessive decalcification by EDTA. Even in optimally fixed and decalcified tissues, some antigens are difficult to demonstrate. Cyclin D1 and CD4 are problematic in this regard with currently available monoclonal antibodies. Non-specific nuclear staining may occur with a variety of antibodies in tissues that are poorly fixed, excessively decalcified or both. In our experience, the antibodies UCHL-1 (CD45RO), Ber-H2 (CD30) and NB84 are particularly prone to this problem.

*Technical problems due to endogenous enzyme activity and non-specific antibody binding.* Endogenous enzyme activity and non-specific antibody binding can both lead to technical problems. When immunostaining trephine biopsy sections with detection systems based on horseradish peroxidase, particular attention must be paid to blockade of endogenous peroxidase activity. Granulocytes express abundant peroxidase activity that is not destroyed by fixation or processing. Use of methanolic hydrogen peroxide is satisfactory but longer incubation (e.g. 30 minutes, compared with 15 minutes for most other tissues) is helpful. Because the solution oxidizes rapidly, replacement with freshly prepared methanol/H₂O₂ at intervals during incubation is helpful. Addition of sodium azide to the final chromogenic substrate provides additional peroxidase blockade in difficult cases.

When using an alkaline phosphatase–anti-alkaline phosphatase detection system, there is rarely any problem from endogenous alkaline phosphatase activity, since the enzyme is largely destroyed during processing. A weak background blush may be seen in some cases, due to residual activity in stromal cells. This can be inhibited by adding levamisole to the chromogenic substrate.

Occasionally, endogenous biotin expression can cause non-specific staining when avidin–biotin or streptavidin–biotin detection systems are used. This is found particularly when using antibody AA1 to demonstrate mast cell tryptase. Such activity can be blocked by sequential incubation of sections with saturating solutions of avidin and biotin prior to immunostaining.

Non-specific binding of antibodies to protein residues in the tissue, which may be a particular problem with polyclonal antisera, can be blocked by pre-incubation of sections with bovine serum albumin or normal human serum. Background serum staining with anti-immunoglobulin light and heavy chain antibodies may be reduced by use of wet heat methods for antigen retrieval rather than proteolysis.

*Problems of interpretation.* Other problems in interpretation can occur. As mentioned above, immunostaining for antigens such as CD4 and cyclin D1 can be suboptimal and results need to be interpreted with care. Positive controls, with known reactivity, should always be performed to ensure that the technique has worked satisfactorily. It may be necessary to repeat the staining in some cases. It should be noted that cyclin D1 expression in mantle cell lymphoma is nuclear (and only a proportion of cells are positive). This nuclear staining should not be confused with non-specific weak cytoplasmic staining that is sometimes observed in other neoplastic lymphocytes.

Expression of CD5 by neoplastic B cells in chronic lymphocytic leukaemia (CLL) and mantle cell lymphoma is much weaker than the constitutive expression of this antigen by T cells. For this reason, it is important to ensure that the dilution of the antibody used is optimal for the detection of weakly expressed antigens on neoplastic cells. Similarly, it is important to use for comparison a positive control that represents one of these lymphomas, rather than normal tissue. The detection of neoplastic B cells in CD5-immunostained bone marrow trephine biopsy sections requires careful evaluation of sections since such cells are often present among a background population that contains numerous, strongly stained, non-neoplastic T cells.

Detection of BCL2 expression by cells in B-cell lymphoid infiltrates in bone marrow is seldom useful because of the abundance of non-neoplastic, BCL2-positive T cells, the rarity of follicle formation.
and the positive reactions that can be observed not only in follicular lymphoma but also in other B-cell lymphomas.

**Cytogenetic analysis**

Specific, non-random chromosomal abnormalities are a common finding in haematological neoplasms and often play a central role in pathogenesis. In addition, some haematological neoplasms are defined more precisely by the presence of specific chromosomal abnormalities than by haematological or histological features. For example, a specific subtype of AML, which can be designated M4Eo/inv(16)(p13q22)/CBFβ-MYH11 fusion AML, is better defined by the presence of inversion of chromosome 16 than by the cytological or histological features of acute myelomonocytic leukaemia with eosinophilia. The presence of certain chromosomal abnormalities also offers prognostic information. For example, in AML, the presence of t(8;21)(q22;q22), t(15;17)(q22;q21) or inv(16)(p13q22) is indicative of a better prognosis. Cytogenetic analysis can also help in distinguishing a neoplastic from a reactive process, as when the demonstration of a clonal cytogenetic abnormality provides evidence that a case of ‘idiopathic’ hypereosinophilic syndrome is actually eosinophilic leukaemia.

Classical cytogenetic analysis can be performed only on cell suspensions such as those obtained from peripheral blood or bone marrow [20]. Cytogenetic analysis for investigation of suspected haematological neoplasms involves the examination of metaphase spreads which can be prepared from blood or from bone marrow aspirates either as direct preparations, in tumours with a high proliferative fraction, or after a preliminary period of culture either with or without mitogens. Cells are arrested in metaphase by exposure to a spindle poison such as colcemid. After cell lysis the chromosomes are visualized with stains such as Giemsa or quinacrine mustard (a fluorescent agent). Individual chromosomes are identified by their size, by the position of the centromere and by their banding pattern (the sequence of light and dark bands apparent after staining). The findings may be illustrated by a karyogram, an ordered array of chromosomes (Fig. 2.21). Alternatively, they may be expressed as a karyotype. For example, the karyotype 47,XY,+8 [18], 46,XY [2] from the bone marrow of a male patient indicates the presence of a clone of cells with trisomy 8; of the 20 metaphases examined, two were normal.

Cytogenetic analysis has a major role in haematological diagnosis. Applications include:

1. Confirmation of diagnosis, e.g. by demonstration of t(15;17)(q22;q21) in M3 variant AML;
2 detection of chromosomal rearrangements that are indicative of good or bad prognosis and should be considered in the choice of treatment, e.g. demonstration of hyperdiploidy, indicative of good prognosis in ALL;

3 confirmation of a neoplastic process when this is otherwise difficult, e.g. in the hypereosinophilic syndrome or in lymphocytosis with large granular lymphocytes;

4 monitoring treatment, e.g. by estimation of the number of Philadelphia chromosome-positive metaphases when a patient with chronic granulocytic leukaemia is being treated with interferon;

5 post-transplant monitoring, e.g. by study of sex chromosomes in a sex-mismatched allogeneic stem cell or bone marrow transplant recipient;

6 diagnosis of therapy-related AML and MDS (see Chapter 4); and

7 confirmation of a constitutional abnormality that may underlie the development of a haematological malignancy, e.g. detection of trisomy 21 in a child with acute megakaryoblastic leukaemia in whom Down’s syndrome is suspected, or demonstration of sensitivity to clastogenic agents, confirming a diagnosis of underlying Fanconi’s anaemia in a patient presenting with AML.

Problems and pitfalls

Metaphase spreads in leukaemias are often of poor quality so that the characterization of an abnormality can be difficult. In addition, some specific chromosomal rearrangements, e.g. t(12;21)(p12;q22) in ALL, are difficult to detect by karyotypic analysis while others are impossible; for these rearrangements, molecular genetic techniques are required.

Cytogenetic analysis fails or yields too few metaphases for adequate analysis in a proportion of cases of acute leukaemia. Inappropriate techniques may mean that there is selection for residual normal cells, e.g. if direct examination rather than preliminary culture is used for the investigation of acute promyelocytic leukaemia.

When there is only partial replacement of the marrow by a neoplastic clone, e.g. in the MDS, a cytogenetically abnormal clone may be present but may not be identified if insufficient metaphases are examined.

In some slowly growing tumours it is not possible to obtain suitable metaphase preparations and metaphases may represent residual normal cells rather than neoplastic cells. This is often the case, for example, in CLL.

Molecular genetic analysis

Cytogenetic analysis has been part of the diagnostic assessment of haematological neoplasms for many years. More recently, techniques such as Southern blot analysis and PCR have allowed molecular genetic events associated with such chromosomal abnormalities to be studied. These techniques have also led to the detection of additional genetic abnormalities in haematological diseases. They are being used increasingly for diagnosis and follow-up of patients on a routine basis.

In addition, molecular genetic analysis can be used to establish clonality; this is particularly valuable in lymphoid neoplasms, where antigen receptor gene rearrangements provide unique clonal markers for neoplastic cell populations. Molecular genetic techniques are most readily applicable to peripheral blood or bone marrow aspirates but modified techniques suitable for application to trephine biopsies are being developed currently.

New concepts with regard to somatic hypermutation of antigen receptor (IGH and TCR) variable region genes in lymphoid cell maturation are emerging currently from molecular genetic analysis. Knowledge of such mechanisms in T-cell maturation is much less advanced than it is for the B-cell lineage. Basic understanding of the somatic mutation processes underlying affinity maturation of immunoglobulin molecules, which occur within germinal centres, allows distinction between lymphomas derived from pregerminal centre (non-mutated), germinal centre (hypermutated with evidence of ongoing acquisition of additional mutations) and postgerminal centre (hypermutated with no ongoing mutation) lymphoid cells. For example, subcategories of CLL have been described which differ by virtue of showing pregerminal centre (60%) and postgerminal centre (40%) patterns of IGH hypermutation.

Molecular analysis can also be used to identify viral DNA or RNA in blood or bone marrow (see Chapter 3).
Fluorescence and other in situ hybridization techniques

*In situ* hybridization (ISH) is a molecular genetic technique although it can also be regarded as an extension of conventional cytogenetics. It is based on the hybridization of a labelled probe to interphase nuclei or metaphase spreads. The technique can employ fluorescence microscopy (fluorescence *in situ* hybridization or FISH), enzymatic methods [21,22] or a radioactive label. Probes, consisting of synthetic DNA in various forms, visualized with the aid of fluorochromes, enzymes or radio-isotopes, can be used to detect numerical abnormalities of chromosomes or the presence of various chromosomal rearrangements. Target DNA may be identified by means of a probe conjugated to a fluorochrome. Alternatively, binding of the probe to target DNA can be identified by means of hybridization of the primary probe to complementary bases in a second complementary probe that also contains a reporter molecule [20]. Following stringency washes performed to remove excess probe, binding of the probe to its target DNA is detected by means of complexing of the reporter molecule in the second probe with a reporter-binding molecule conjugated either to a fluorochrome or to an enzyme such as peroxidase or alkaline phosphatase. By using different fluorochromes or two enzymes it is possible to identify simultaneously multiple (typically two or three) different specific DNA sequences in a single preparation of cells. Direct fluorescence methods are more rapid than indirect and give less non-specific background staining, whereas indirect fluorescence methods generally give a stronger signal. Enzymatic methods are currently less used but have the advantage that a fluorescence microscope is not needed.

Fluorescence and other ISH techniques are applicable to: (i) films of blood or bone marrow cells; (ii) imprints of trephine biopsy specimens; (iii) cytocentrifuge preparations; and (iv) films of cells that have been cultured with or without mitogens. These techniques have the particular advantage over classical cytogenetic analysis that they can be applied not only to metaphase spreads but also to interphase nuclei. Chromosome abnormalities can therefore be detected in neoplastic cells that do not readily enter mitosis, such as the neoplastic cells of CLL and multiple myeloma. In addition, ISH can be applied to blood or bone marrow films previously stained with a Romanowsky stain or by immunocytochemistry, thus permitting correlation of cytological and immunophenotypic features with karyotypic information. Obviously, unlike cytogenetic analysis, ISH permits visualization of only restricted areas of individual chromosomes rather than giving a global view of the entire chromosome complement within a cell.

The probes most often employed in ISH techniques are: (i) repetitive sequence centromeric probes (available for all chromosomes and applicable to cells in interphase or metaphase); (ii) whole chromosome paints (available for all chromosomes but applicable only to cells in metaphase); and (iii) specific sequence probes, including those identifying oncogenes, cancer-suppressing genes and the breakpoints of recurring translocations (applicable to cells in interphase or metaphase).

Numerical abnormalities of chromosomes can be detected either with centromeric probes or with whole chromosome paints. When such probes are used, normal cells have two separate fluorescent signals. If there is an abnormality of chromosome number, the number of signals will vary. For example, in trisomy 12, FISH with a centromeric probe for chromosome 12 shows three signals per cell whereas, in monosomy 7, FISH with a centromeric probe for chromosome 7 shows only one signal per cell (Fig. 2.22). If using centromeric probes, rather than whole chromosome paints, care must be taken in making assumptions that gain or loss of a signal represents gain or loss of an entire chromosome target.

Several strategies can be used for detecting specific translocations or other rearrangements. Use of a single probe spanning a specific chromosomal breakpoint is useful when multiple chromosomal partners can disrupt a gene of interest, as is the case with *MLL* and *BCL6*. When both partner chromosomes are predictable a variety of techniques can be used. Whole chromosome paints can be used for the two chromosomes of interest. Alternatively, two probes can be selected which bind to specific oncogenes or bind to the two chromosomes of interest at a site close to the expected breakpoint. The two probes are labelled with different fluorochromes. In normal cells there will be two separate signals of different colours whereas, in cells in which a translocation
has occurred, the two colours come together on a single chromosome (Fig. 2.23). Alternatively, probes spanning the expected breakpoint on each chromosome can be used so that, if a translocation has occurred, the signals are split. Each abnormal chromosome then has two adjacent signals of different colours, while the two remaining normal chromosomes have a single colour signal (Fig. 2.24); this technique is sometimes referred to as D-FISH, signifying double fusion FISH. It is also possible to combine one probe spanning an expected breakpoint with another that is adjacent to the second breakpoint. A single probe spanning one of the breakpoints can also be used, a split signal being consistent with a translocation. In this case it is sometimes necessary to demonstrate that three signals represent a normal and a split signal rather than trisomy (Fig. 2.25). Triple colour FISH permits the use of a single probe identifying a sequence on one of the chromosomes implicated together with two separate probes identifying genes on either side of the breakpoint on the second chromosome. The latter two signals are dissociated when the relevant translocation occurs (Fig. 2.26).

**Fig. 2.22** Diagrammatic representation illustrating the principles of FISH, using a centromeric probe for the identification of trisomy or monosomy. (a) Centromeric probe for chromosome 12 in chronic lymphocytic leukaemia showing four trisomic cells and one disomic cell. (b) Centromeric probe for chromosome 7 in myelodysplastic syndrome showing all cells to have monosomy 7.

**Fig. 2.23** Diagrammatic representation illustrating the principles of FISH, using two oncogene probes to detect a translocation. Probes for BCR (red) and ABL (green) have been used. In the normal cell (left) there are four separate signals. In the cell from a patient with t(9;22)(q34;q11) associated with chronic granulocytic leukaemia (right) there are single normal BCR and ABL signals and a double red plus green signal where the two oncogenes have been juxtaposed. The red–green signal is yellow.
difficult to recognize on classical cytogenetic analysis if preparations are suboptimal, can be detected; large numbers of cells can be scanned so that a low frequency abnormal population can be recognized; and deletion of cancer-suppressing genes or amplification of oncogenes can be demonstrated.

Fluorescent immunophenotyping and interphase cytogenetics (FICTION) is a technique combining immunophenotyping with FISH. It can be used, for example, to demonstrate that trisomy 12 in CLL can occur in only a proportion of the clonal lymphocytes.

A recent development of FISH is spectral karyotyping (SKY) in which a complex panel of probes applied simultaneously permits the identification of all chromosomes in metaphase preparations.

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**Fig. 2.24** Diagrammatic representation illustrating the principles of FISH, using two oncogene probes, both of which are split in a given translocation, to detect the translocation. Probes spanning **BCR** (red) and **ABL** (green) have been used. In the normal cell (left) there are four separate signals. In the cell from a patient with t(9;22)(q34;q11) associated with chronic granulocytic leukaemia (right) there are single normal **BCR** and **ABL** signals and two double red plus green signals (fusion signals are yellow) representing **BCR-ABL** and **ABL-BCR**.

Probes spanning specific breakpoints are also useful for detecting isochromosome formation (Fig. 2.27) and chromosomal inversion. For example, inversion of chromosome 16 can be detected by using a probe that spans one of the breakpoints. When a pericentric inversion has occurred, the signal is split and appears on both the long and short arms of the chromosome in a metaphase spread (Fig. 2.28).

The FISH technique can also be used to identify deletion or amplification of chromosomal material. For example, specific probes can be used to show deletion of a cancer-suppressing gene such as **RB1** or **p53** or amplification of an oncogene such as **MYC**.

An ISH technique has the following advantages over classical cytogenetics:

1. living cells are not required;
2. some abnormalities can be recognized in interphase nuclei, making the technique particularly useful for neoplasms, such as CLL, with a low proliferative rate;
3. a suspected abnormality can be confirmed when morphology of the chromosomes is poor;
4. chromosomal rearrangements, such as inversion(16), which have only subtle alterations in the chromosome banding pattern and are therefore difficult to recognize on classical cytogenetic analysis if preparations are suboptimal, can be detected; large numbers of cells can be scanned so that a low frequency abnormal population can be recognized; and
5. deletion of cancer-suppressing genes or amplification of oncogenes can be demonstrated.

Fluorescent immunophenotyping and interphase cytogenetics (FICTION) is a technique combining immunophenotyping with FISH. It can be used, for example, to demonstrate that trisomy 12 in CLL can occur in only a proportion of the clonal lymphocytes.

A recent development of FISH is spectral karyotyping (SKY) in which a complex panel of probes applied simultaneously permits the identification of all chromosomes in metaphase preparations.

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**Fig. 2.25** Diagrammatic representation illustrating the principles of FISH, using a whole chromosome paint to detect a translocation. (a) In this case, a paint for chromosome 11 has been used. A normal cell (left) shows two signals, whereas a cell from a patient with t(11;14)(q13;q32) associated with mantle cell lymphoma (right) shows three signals representing, respectively, one normal chromosome and two signals representing the two parts of chromosome 11 separated as a result of translocation. (b) To ensure that the three signals do not represent trisomy for chromosome 11, FISH can also be performed with a centromeric probe for chromosome 11; since the centromere is not split in the translocation this shows two signals in both the normal cell (left) and the cell with the translocation (right).
This technique is particularly useful for investigating complex chromosomal abnormalities. Because expensive equipment, reagents and software are required the technique is currently mainly used in research rather than in routine diagnosis.

Techniques for FISH are applicable to a more limited extent to trephine biopsy sections, in which at present they are best restricted to analysis of potential chromosomal gains. For mathematical reasons, analysis of chromosomal loss in tissue sections (in which many nuclei are only partly represented) is complex. Spatial resolution of FISH signals in histological sections is not adequate with current methods to permit routine application of dual colour techniques to demonstrate translocations, although split signals from a probe spanning an expected break-point can be resolved. Some of these limitations can be overcome by applying FISH to cytocentrifuge preparations from intact nuclei or whole cells extracted by proteolysis from thick sections. As for immunohistochemistry, pre-treatments are required to unmask target DNA sequences. Because these vary considerably with different fixation and decalcification
protocols, FISH applied to bone marrow trephine biopsies is still predominantly a research tool.

It should be noted that ISH techniques to detect messenger RNA (mRNA) targets can also be applied to cytological and histological bone marrow preparations. Either enzymes or fluorochromes can be used to identify the target mRNA. Double labelling with two different fluorochromes is possible, e.g. for either $\kappa$ or $\lambda$ mRNA and for either TNF$\alpha$ or IL-1$\beta$ mRNA, in order to determine whether neoplastic light chain-restricted plasma cells are capable of synthesizing these cytokines. In diagnostic practice, ISH for the detection of mRNA is useful for demonstration of $\kappa$- or $\lambda$-expressing plasma cells (Fig. 2.29) and for the detection of viral mRNA, such as Epstein–Barr early RNA (EBER) in Epstein–Barr virus (EBV) infection. Otherwise, its present role is in research.

Problems and pitfalls

FISH and other ISH techniques have various disadvantages:

1. in any single preparation, it is only possible to identify the specific abnormality for which relevant probes are being employed; for example, if cells are being investigated for the presence of t(8;21), other leukaemia-associated translocations such as t(15;17) or t(9;11) will not be detected;
2. secondary abnormalities are not recognized; for

Fig. 2.29 In situ hybridization showing (a) $\kappa$ and (b) $\lambda$ light chain messenger RNA. There are only occasional $\kappa$-positive cells but $\lambda$-positive cells are numerous. Paraffin-embedded, immunoperoxidase-labelled probes $\times160$. 
example, if the proportion of Philadelphia-positive metaphases were being evaluated in a case of chronic granulocytic leukaemia, the presence of an isochromosome of 17q (which might be associated with impending acute transformation) would not be detected;
3 the use of whole chromosome paints does not permit the detection of rearrangements within a single chromosome, such as an inversion, or small deletions;
4 in screening for trisomies and monosomies there are some false-positive findings, caused by accidental co-localization of signals, so that a low frequency of the abnormality being sought cannot be detected reliably; and
5 if neoplastic cells are present in the bone marrow but cannot be aspirated because of associated fibrosis, there will be a failure to detect any relevant cytogenetic abnormality.

The first two of these disadvantages can be circumvented to some extent by the use of multicolour FISH or spectral karyotyping [21].

Southern blot analysis
In this technique DNA is extracted from a fresh, unfixed tissue sample and digested with a panel of restriction endonucleases. Sometimes, when the bone marrow is cellular, unfixed bone marrow films on glass slides may yield sufficient material for analysis. The restriction endonucleases used are enzymes that recognize specific nucleotide sequences between 4 and 10 base pairs in length and cut the DNA molecule wherever their target sequences occur. This results in DNA fragments of variable length that can be separated, according to their size, by gel electrophoresis. These are then transferred by capillary action onto a more solid nitrocellulose membrane to create a Southern blot. Fragments containing the DNA sequences of interest can then be identified by hybridization with a radiolabelled DNA probe followed by autoradiography. One of the major breakthroughs that Southern blot analysis allowed was the detection of clonality in B- and T-lymphoid neoplasms (see below). In addition, rearrangement of a gene arising from translocation or some other acquired chromosomal rearrangement can be detected. This technique is used, for example, for detecting rearrangement of the \textit{MLL} or \textit{BCL6} genes since rearrangement can result from a considerable number of different translocations, not all of which can be detected by alternative techniques. The Southern blot technique is also applicable for the detection of translocations in which there is considerable heterogeneity of breakpoints precluding use of a simple PCR technique.

Southern blot analysis can be used to demonstrate clonal integration of HTLV-I proviral DNA and integration of defective HTLV-I in adult T-cell leukaemia/lymphoma [23].

Problems and pitfalls
The major drawbacks of Southern blot analysis as a diagnostic tool are the requirement for radioactive materials, long turnaround times and the need for relatively large quantities of DNA in the diagnostic sample, which limits the sensitivity of the method. The latter feature limits its applicability to trephine biopsy specimens [24].

The polymerase chain reaction
The polymerase chain reaction (PCR) [25,26] is a method of \textit{in vitro} amplification of a defined DNA target that is flanked by regions of known sequence. The development of this technique has greatly expanded the diagnostic potential of molecular genetics as a result of its ability to generate almost limitless copies of selected target DNA sequences. The technique is 400–4000 times more sensitive than Southern blot analysis. It consists of a repeating cycle of three basic steps (denaturation, primer annealing and elongation), with each cycle potentially leading to a doubling of the amount of the target DNA sequence.

The first step (denaturation) involves heating the DNA sample to around 90°C which causes the double-stranded DNA molecule to separate into two complementary single strands. The next stage (primer annealing) requires short DNA primer sequences that are complementary to the ends of the target DNA segment that is to be amplified. The primer is added to the denatured single-stranded DNA and the sample is cooled. As cooling occurs, the primers anneal (link via the complementary nucleotide sequences) to the single-stranded target DNA. The third step (elongation) requires addition of free nucleotides to the ends of the primer DNA segments, producing complementary copies of each
of the two single-stranded target DNA sequences. This is achieved using a thermostable DNA polymerase. The strands of DNA thus generated are separated from their complementary strands by elevating the temperature again. The cycle is repeated by elevating and lowering the temperature of the container in which the reaction takes place. This process is performed in an automated thermal cycling instrument preset with specific temperatures and times that are optimized for each primer pair. The cycles of denaturing, annealing and elongation are repeated 10–40 times. Amplification is initially exponential since each cycle doubles the amount of DNA template present. In later cycles the rate of increase in the quantity of DNA is closer to linear than to exponential. Nevertheless, very large quantities of the target DNA sequence are generated. The size of the DNA segment can be estimated by electrophoresis, by comparison with known standards (Fig. 2.30). The DNA generated can be directly visualized by staining with ethidium bromide and viewing under UV light. This technique has many advantages over Southern blot analysis for diagnostic use, principally the short turnaround time, the lack of a requirement for radio-isotopes, the ability to amplify very small quantities of target DNA and its applicability to fixed tissue, including archival samples and even stained or unstained blood or bone marrow films scraped off glass slides. Stained slides are, however, less satisfactory than paraffin-embedded tissue because DNA degradation is greater. Techniques are available for use with decalcified paraffin-embedded trephine biopsy specimens [27]; EDTA decalcification rather than formic acid decalcification is required to ensure that DNA is not degraded. The amplification achieved by PCR makes the technique very sensitive. The DNA fragments produced are suitable for sequencing. Because of these advantages PCR, or its modification reverse transcriptase-PCR (RT-PCR), has replaced Southern blot analysis for most diagnostic applications. To distinguish it from RT-PCR, PCR is sometimes referred to as genomic PCR or DNA-PCR.

**In situ** PCR is a modification of the PCR technique performed on tissue sections pre-treated with a proteinase to facilitate entry of primers [28].

PCR can be used for investigation of clonality (by amplification of *IGH* or *TCR* genes), for detection of rearrangement of genes resulting from acquired cytogenetic abnormalities and for detection of bacterial or viral sequences (for example, sequences of *Mycobacterium tuberculosis* in a bone marrow aspirate).

PCR can be used to demonstrate clonality of lymphoid cells by amplification of Epstein–Barr virus

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**Fig. 2.30** Polyacrylamide gel stained with ethidium bromide and visualized with ultraviolet light showing PCR amplification of the 643 base pair BRCA1 exon 11A (top panel) and 482 base pair BRCA2 exon 11B (bottom panel). Lane 1 contains size standards and lanes 2–10 contain amplified samples of DNA extracted from EDTA-decalcified bone marrow trephine biopsy specimens. (By courtesy of Mrs Caroline Wickham, Exeter.)
DNA. Because of a variable number of terminal repeats in the circular episomal forms of the virus a polyclonal lymphoid population with a variety of forms of the virus can be distinguished from a monoclonal population with a single form. This can be useful for establishing clonality of EBV-induced natural killer cell lymphomas for which there are usually no alternative techniques for demonstrating clonality.

Problems and pitfalls

Great care is required to avoid contamination during PCR analysis as even the smallest quantity of DNA with a sequence complementary to the primers will be amplified. Meticulous technique is required.

Although PCR is applicable to archival material, poor preservation of DNA can lead to negative results [24]; amplification of a constitutional gene, for example G6PD, BCR, ABL or the β globin gene, can be used to control for DNA degradation. Preservation of DNA is determined by the fixative used and the method of processing. Formalin fixation has been found to give optimal results but other fixatives, e.g. B5, can be used [24]. As noted above, DNA from archival glass slides is often of inferior quality.

Negative results can result from sampling error when tissue infiltration is focal.

Variant breakpoints may be missed if appropriate primers are not used. For example, in testing for BCL2 rearrangement in follicular lymphoma it is necessary to screen for breakpoints in the minor cluster region (MCR) as well as the major breakpoint region (MBR).

In some neoplasms, PCR is an insensitive diagnostic technique to amplify fusion genes because of the wide scatter of breakpoints. For example, in mantle cell lymphoma, genomic PCR detects only a little over 50% of cases, specifically those cases with a chromosome 11 breakpoint in the major translocation cluster [26].

Reverse transcriptase-polymerase chain reaction

PCR is applied to genomic DNA. Because of the presence of introns, a gene may be very large so that satisfactory amplification is difficult. This problem can be circumvented by the use of mRNA as the starting material since the introns will have been excised and the segment to be amplified is shorter. The mRNA must first be transcribed into complementary DNA by means of reverse transcriptase. Amplification can then proceed, as in PCR of genomic DNA. RT-PCR is now very commonly employed for the detection of leukaemia and lymphoma-related fusion genes.

RT-PCR can also be used for the detection of viral mRNA (see Chapter 3).

Modifications of PCR and RT-PCR techniques

Modifications of PCR and RT-PCR techniques (Table 2.8) can increase sensitivity, specificity and usefulness and permit an approximate quantification of the number of copies of the fusion gene present. Among these modifications is multiplex PCR, in which a number of sets of primers are used to amplify simultaneously two or three different fusion genes. This makes it possible, for example, to screen simultaneously for the three fusion genes found in the three subtypes of AML associated with a better prognosis: AML1-ETO, PML-RARα and CBFβ-MYH11. The development of various techniques for quantification of the amount of target DNA or mRNA present means that PCR and RT-PCR are now becoming useful for the detection of minimal residual disease and for monitoring the response to therapy. One application of quantitative methods is in monitoring BCR-ABL during interferon therapy or following transplantation for chronic granulocytic leukaemia. In real-time PCR (RQ-PCR), the PCR technique results in displacement of a fluorogenic product-specific probe which is degraded during the reaction, generating a fluorescent signal.

Problems and pitfalls

RT-PCR requires intact RNA and is therefore not a suitable technique for use with most archival material. False-negative results can occur if there is degradation of RNA and amplification of a control mRNA sequence is therefore advised.

As for genomic PCR, the high sensitivity of the technique means that there is a risk of false-positive results if contamination is permitted to occur.
Multicentre studies have sometimes shown a high rate of false-positive reactions so that inclusion of a negative control in each assay is considered to be critical [29]. RT-PCR techniques are poorly standardized and quality control is not optimal so that different laboratories produce divergent results in a significant proportion of cases [26].

Since RT-PCR uses a small tissue sample negative results can occur when infiltration, e.g. of a trephine biopsy specimen, is focal. Sometimes microscopy permits the detection of infiltration by lymphoma when results of RT-PCR are negative [24].

It must also be noted that sensitive techniques have detected gene rearrangements typical of leukaemia and lymphoma in people who do not have an identifiable neoplastic condition. This has been noted for **BCR-ABL**, **NPM-ALK**, **AF4-MLL** and **BCL2** rearrangement, characteristic, respectively, of chronic granulocytic leukaemia, large cell anaplastic lymphoma, acute lymphoblastic/biphenotypic leukaemia of infants and follicular lymphoma.

There are also problems in knowing when the detection of minimal residual disease is clinically significant. Detection of a residual clonal abnormality for a year or more after cessation of treatment is sometimes compatible with continued disease-free survival. This has been noted for **AML-ETO**, **MYH11-CBFβ**, **E2A-PBX** and **IGH** gene rearrangements [26,30].

**Other molecular genetic techniques**

Other molecular genetic techniques are rarely used in routine diagnosis but have research uses. They include:

1. northern blot analysis for investigation of RNA;
2. western blot analysis for investigation of proteins including the 'oncoproteins' that are the products of fusion genes;
3. comparative genomic hybridization (CGH) to detect segments of chromosomes that are under- or over-represented in leukaemic cells; and
4. DNA sequencing, which is necessary for cloning of new genes.

**The application of molecular genetic techniques in the investigation of leukaemia and lymphoma**

Molecular genetic techniques have two principal applications in the investigation of haematological neoplasms. Firstly, they can be used to demonstrate
that a monoclonal population is present and can give information on the nature of the lymphoid cell in which a mutation occurred (see below). Secondly, they can be used to demonstrate the presence of fusion genes or fusion RNA transcripts that are known to be associated with specific neoplasms. Demonstration of clonality is mainly done by investigation of \textit{IGH} or \textit{TCR} genes, although demonstration of rearrangement or mutation of any gene could be used as a marker of clonality.

\textbf{Detecting clonal immunoglobulin and T-cell receptor rearrangements}

Clonal populations of B or T lymphocytes can be detected by demonstrating clonal rearrangements of \textit{IGH} or \textit{TCR} genes using Southern blot analysis or PCR. The basic principle underlying the technique is similar since \textit{TCR} and \textit{IGH} genes are both made up of a number of variable (V), diversity (D) and joining (J) regions, present in germline DNA, which rearrange during lymphocyte development to produce a functioning gene. During rearrangement, single V, D and J regions are combined with a constant (C) region and simultaneously nucleotides (known as N) are added and removed between the V, D and J regions. This results in a unique DNA sequence in a cell and its progeny. Rearrangement of \kappa and \lambda genes is similar but these genes lack a D region.

Using Southern blot analysis, probes that detect either part of the \textit{IGH} J region or the \textit{TCR} \beta or \gamma chain genes are used to detect clonal rearrangements. In polyclonal populations of lymphocytes, there are a large number of different rearrangements and no single discrete band can be visualized. In a clonal population, since all the cells share the same rearrangement, there is a single discrete band on electrophoresis which is separate from the germline band.

The technique for detecting clonal rearrangements using PCR is somewhat different. Primers with sequences complementary to segments of the \textit{IGH} V and J regions that are relatively constant are used to amplify part of the rearranged \textit{IGH} gene. Differences in the numbers of nucleotides (N) inserted between the V and J regions during rearrangement result in multiple different sized segments of DNA being amplified in polyclonal populations, so that no discrete band is visualized. In clonal populations, there is amplification of a single rearranged fragment which appears as a discrete band separate from the germline band on electrophoresis. The same principle is used to detect T-cell clones using probes complementary to sequences in the \textit{TCR} \gamma and \beta chains.

Molecular genetic analysis has led to new concepts with regard to somatic hypermutation of antigen receptor (\textit{IGH} and \textit{TCR}) variable region genes in lymphoid cell maturation, with potential relevance to our understanding of the origins and behaviour of all lymphomas. Knowledge of such mechanisms in T-cell maturation is much less advanced than it is for the B-cell lineage.

\textbf{Problems and pitfalls}

Although PCR is more sensitive than Southern blotting at detecting small clonal populations, it will not detect all rearrangements. Approximately 80% of B-cell neoplasms will show clonal rearrangements on PCR using primers for \textit{IGH} V regions, whereas the great majority will have a rearrangement detectable by Southern blot analysis. Hence caution is required in interpreting negative PCR results. The likelihood of detecting \textit{IGH} gene rearrangement by PCR varies between different types of lymphoma, in one study ranging from around 40% with follicular lymphoma to around 80% with mantle cell and small lymphocytic lymphomas [26]. The negative results in follicular and certain other lymphomas are consequent on the high rate of somatic mutation which is responsible for a failure of consensus primers to bind to rearranged genes [24].

It should also be noted that rearrangement of \textit{IGH} and \textit{TCR} genes is not lineage-specific. Rearrangement of \kappa or \lambda light chain genes is much more specific for the B-lymphocyte lineage than \textit{IGH} gene rearrangement. Inappropriate gene rearrangement is quite frequently seen in acute lymphoblastic leukaemia and occurs, although less often, in lymphomas of mature T and B cells. In addition, \textit{IGH} or \textit{TCR} gene rearrangements are sometimes found in acute myeloid leukaemia.

It is likewise important to recognize that, although the detection of a clonal rearrangement usually indicates a neoplastic process, clonal \textit{IGH} and \textit{TCR} gene rearrangements have been detected in some reactive conditions.
Detection of leukaemia/lymphoma-associated fusion genes

As discussed earlier, many haematological neoplasms are associated with a specific non-random chromosomal abnormality. The genes involved in many of these rearrangements have been identified, permitting the use of molecular genetic techniques for their detection. Some translocations can be detected by PCR, by employing specific primers with sequences complementary to segments of DNA that flank the chromosomal breakpoints. The intervening segment across the breakpoint will only be amplified if the translocation is present. However, this technique is only applicable if the breakpoint occurs in a relatively constant position and the fusion gene is not too long. Many more translocations can be detected by RT-PCR. Translocations that can be detected by PCR or RT-PCR include t(9;22)(q34;q11) in chronic granulocytic leukaemia and some acute lymphoblastic leukaemias, t(14;18)(q32;q21) in follicular lymphoma, t(2;5)(p23;q25) in anaplastic large cell lymphoma and various translocations or inversions associated with acute myeloid leukaemia including t(8;21)(q22;q22), t(15;17)(q22;q21) and inv(16)(p13q22).

Advantages of PCR and RT-PCR are that they give a more rapid result than conventional cytogenetic analysis and do not require viable cells for metaphase preparations. The highly sensitive nature of PCR analysis also means that this technique can be used to detect very small numbers of neoplastic cells; consequently, the method can be used for the detection of minimal residual disease and early relapse. However, it should be noted that, using these sensitive techniques, fusion genes characteristic of leukaemia or lymphoma are sometimes detected in normal tissues or tissues showing only reactive changes. For example, the rearrangement characteristic of follicular lymphoma has been detected in tonsils removed surgically for reactive conditions.

Problems and pitfalls

Disadvantages, other than the possibility of contaminating DNA being amplified, include the fact that not all rearrangements can be detected. This is because of the necessity for a chromosomal abnormality to have been fully characterized so that specific primers for each breakpoint can be designed. It should also be noted that, in contrast to standard cytogenetic analysis, molecular genetic techniques permit the detection only of those abnormalities that are being specifically sought. Nevertheless, many chromosomal rearrangements can be detected by PCR and RT-PCR and some such rearrangements are sufficiently common that these techniques are very practical for the rapid and precise categorization of cases of acute leukaemia and for confirming the diagnosis of certain categories of lymphoma.

Ultrastructural examination

Ultrastructural examination, in which the structure of cells is studied by electron microscopy, can be applied to peripheral blood and bone marrow but is little used in routine diagnostic haematology. The advances in immunophenotyping have rendered electron microscopy redundant in identifying M0 and M7 AML. It remains of some use in identifying small Sézary cells (Fig. 2.31) and in making a precise diagnosis in congenital dyserythropoietic anaemia.

Ultrastructural examination can also be applied to trephine biopsy specimens but it is rarely necessary for diagnosis. It can be used for the detection of Birbeck granules for confirming the diagnosis of Langerhans cell histiocytosis but immunohistochemistry, including the application of a CD1a monoclonal antibody, is an alternative more readily available technique that can be used for this purpose.

Bone marrow culture for assessment of haemopoietic progenitor cell numbers

Short- and long-term culture techniques for haemopoietic cells have found extensive use in research but have only limited roles in the diagnostic assessment of bone marrow disorders.

Short-term culture

The major current use of short-term culture in clinical practice is for assessment of harvested bone marrow or peripheral blood stem cells prior to their use for engraftment.

Short-term haemopoietic cultures are performed using cells suspended at a known starting concentration in methyl cellulose or agar supplemented
with culture medium, fetal bovine serum and
growth-promoting substances such as granulocyte–
macrophage colony-stimulating factor (GM-CSF),
erthropoietin and thrombopoietin. They are then
incubated at 37°C for 14 days, in a humidified
atmosphere containing 5% CO₂. Depending upon
the precise conditions of the assay, multipotential
cells in the patient’s (or donor’s) sample will form
colonies containing variable proportions of differenti-
at ing cells of various haemopoietic lineages. The
starting cells are seeded at a sufficiently low con-
centration that individual colonies localized around
each single multipotential parent cell can be visual-
ized separately from any neighbouring colonies.
The colonies can then be counted at low power
magnification by standard light microscopy.

The most primitive multipotential cell type usu-
ally assayed in such a culture system is the mixed
colonies are seen by light microscopy because of the
orange–red colour of their cytoplasmic haemoglobin.
Apart from the use of culture systems as assays for predicting haemopoietic function in transplantation, spontaneous colony formation (i.e. colony formation with no growth factor supplementation) is used by some laboratories to assist in diagnosing chronic myeloproliferative disorders. Spontaneous megakaryocyte colony-forming unit (CFU-Meg) activity, spontaneous BFU-E activity, or both, is demonstrable in peripheral blood or bone marrow cells from many patients with essential thrombocythaemia. Spontaneous BFU-E also form from peripheral blood and bone marrow cells of patients with polycythaemia rubra vera. Blood or marrow cells from normal individuals and patients with reactive thrombocytosis or secondary polycythaemia do not usually show spontaneous BFU-E activity. However, they may occasionally give rise to CFU-Meg. This potential for false-positive results and a relatively high false-negative rate in true cases of essential thrombocythaemia and polycythaemia rubra vera, together with the cumbersome nature of the assays, have limited the diagnostic application of short-term colony-forming assays.

**Long-term culture**

Long-term cultures, in which haemopoietic precursor cells are seeded onto layers of pre-grown marrow stroma, can potentially assess the activity of stem cells even more primitive than those which survive in short-term cultures. Assay of seeded cells is achieved, once they have adhered to the stroma, by replacing liquid medium with methylcellulose or agar-containing semi-solid medium and assessing colony formation as above. This system is known as the long-term culture-initiating cell (LTC-IC) assay. It is mainly employed in research, despite its possible value in predicting engraftment. Long-term cultures have also been used for ex vivo purging and expansion of haemopoietic cells for engraftment but these approaches have yet to find widespread clinical application.

**Bone marrow culture for micro-organisms**

Bone marrow culture for micro-organisms, for example mycobacteria, *Leishmania donovani* or *Histoplasma capsulatum*, can be of use in diagnosis and will be discussed in the next chapter.

**References**

SPECIAL TECHNIQUES