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The role of cytology, cytochemistry, immunophenotyping and cytogenetic analysis in the diagnosis of haematological neoplasms

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Summary Cytology, cytochemistry, immunophenotyping and cytogenetic analysis have specific roles in the diagnosis and management of various haematological neoplasms. Careful examination of Romanowsky-stained films of blood and bone marrow is fundamental in all haematological diagnosis and, when considered together with clinical and haematological features, indicates which of the more specialized techniques are most likely to be useful. The major role of cytochemistry is in the diagnosis of acute myeloid leukaemia and the myelodysplastic syndromes. The major role of immunophenotyping is in the diagnosis of the chronic lymphoproliferative disorders and of acute leukaemia which is not obviously myeloid. Cytogenetic analysis has a role in confirming the diagnosis of chronic granulocytic leukaemia and gives important supplementary information in the acute leukaemias and the myelodysplastic syndromes.

Keywords Cytochemistry, immunophenotyping, cytogenetics, leukaemia

Introduction

In recent years, immunophenotyping and cytogenetic analysis have become increasingly important in characterizing haematological neoplasms whilst the role of cytochemistry has diminished. This guideline discusses the place of these three methods of investigation and outlines the essential tests needed for clinically meaningful diagnosis. Details of recommended techniques are given elsewhere (ICSH 1985, 1993; BCSH 1994a,b; Rooney & Czepulkowski 1992). The terminology and classifications used in this guideline are those recommended by the French–American–British Cooperative Group. Our recommendations are summarized in Tables 1–3 and are discussed in more detail in the following text.

Cytology

Despite advances in other areas, careful microscopical examination of Romanowsky-stained peripheral blood (PB) and bone marrow (BM) films remains fundamental in haematological diagnosis. Microscopy alone may provide a definitive diagnosis of acute myeloid leukaemia (AML) and the myelodysplastic syndromes (MDS) and a provisional cytological diagnosis of acute lymphoblastic leukaemia

Table 1. Essential and useful techniques

Essential techniques
Cytology
Cytochemistry
Either Sudan black B or myeloperoxidase reaction
Either alpha-naphthyl acetate esterase or a combined reaction such as alpha-naphthyl acetate esterase plus naphthol AS-D chloroacetate esterase
Perls' stain for haemosiderin
Immunophenotyping
Use of primary and secondary panels of antibodies for diagnosis of the acute leukaemias and the chronic lymphoproliferative disorders as recommended in previous guidelines (BCSH 1994a,b)
Useful techniques
Cytochemistry
Neutrophil alkaline phosphatase reaction
Tartrate-resistant acid phosphatase reaction
Immunophenotyping
Use of panels of selected antibodies for the diagnosis of large granular lymphocyte leukaemia and hairy cell leukaemia

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Table 2. Role of specific tests

Test	Role
Cytology	Examination of Romanowsky-stained films is essential in the diagnosis of all haematological neoplasms
Cytochemistry	
MPO/SBB	Essential in acute leukaemia unless myeloid differentiation is obvious; essential in MDS unless the diagnosis of RAEB-t has been established from the Romanowsky-stained film; useful in acute transformation of chronic granulocyte leukaemia
NSE/CE	Essential in acute leukaemia if M2, M4 or M5 AML is suspected
NAP	Useful in chronic myeloid leukaemias if cytogenetic and DNA analysis are not available; sometimes useful in the diagnosis of other myeloproliferative disorders; useful in the diagnosis of PNH developing in aplastic anaemia
TRAP	Useful in the diagnosis of hairy cell leukaemia but not essential if detailed immunophenotyping is available and/or trephine biopsy is characteristic
Immunophenotyping	Essential in acute leukaemias unless obviously myeloid; useful in acute transformation of chronic granulocytic leukaemia unless blast cells are clearly myeloid; can be used in the diagnosis of PNH; essential in lymphoproliferative disorders
Cytogenetic analysis	Strongly recommended in all cases of acute leukaemia; important if the diagnosis of MDS is suspected; useful in MDS in indicating prognosis; strongly recommended in CGL but not generally useful in other MPD; useful in confirming the diagnosis of specific lymphoproliferative disorders

MPO, myeloperoxidase; SBB, Sudan black B; NSE, non-specific esterase; CE, combined esterase; NAP, neutrophil alkaline phosphatase; TRAP, tartrate-resistant acid phosphatase; MDS, myelodysplastic syndromes; AML, acute myeloid leukaemia; PNH, paroxysmal nocturnal haemoglobinuria; CGL, chronic granulocytic leukaemia; MPD, myeloproliferative disorders

(ALL) (which requires immunophenotypic confirmation). Microscopy is also crucial in the diagnosis of chronic myeloid leukaemias and is necessary in the diagnosis of other myeloproliferative disorders (MPD). In the lymphoproliferative disorders (LPD), microscopy is important but may be unreliable if not supplemented by immunophenotyping.

Cytology can fail in a number of situations. For example, splenic lymphoma with villous lymphocytes, follicular lymphoma and the small cell variant of T-prolymphocytic leukaemia can all be misdiagnosed as chronic lymphocytic leukaemia. Similarly, not all cases of mature B-cell ALL have an L3 morphology and rare cases with L3 morphology are not B-ALL.

Although cytology is usually reliable in the diagnosis of both Ph-positive and Ph-negative (BCR-ABL-positive) chronic granulocytic leukaemia (CGL), consistent diagnosis of the other chronic myeloid leukaemias is not always achieved. Nevertheless, at present, cytology is the best technique available for diagnosing and classifying the Ph-negative chronic myeloid leukaemias.

Certain PB characteristics are useful in distinguishing between atypical chronic myeloid leukaemia (aCML) and chronic myelomonocytic leukaemia (CMML) (Galton 1992; Bennett *et al.* 1994). Anaemia, thrombocytosis and monocytosis are more common in aCML than in CGL, eosinophilia and basophilia are less consistently present and granulocytic dysplasia is common. Atypical chronic myeloid leukaemia differs from CMML in that there are significant numbers of immature granulocytes in the PB, often more than 15% and almost always more than 5%. Eosinophilia and basophilia are uncommon in CMML. The monocyte count does not, by itself, confirm a diagnosis of CMML since it exceeds 1×10^9/l not only in CMML but also in many cases of aCML.

Erroneous interpretation of specialized investigations often results from neglect of basic microscopy. Such investigations should therefore only be interpreted in the context of clinical, haematological and cytological features. Before proceeding to more specialized tests, it is important to have a preliminary assignment of the particular case to the broad groups of acute leukaemia, MDS, MPD or LPD.

Cytochemistry

Essential cytochemical tests are: (a) myeloperoxidase (MPO) or Sudan black B (SBB); (b) non-specific esterase (NSE) (e.g. alpha-naphthyl acetate esterase) or combined esterase (CE) (e.g. alpha-naphthyl acetate esterase plus naphthol AS-D chloroacetate esterase); and (c) Perls' stain.

Some cases of AML M1 can be identified cytologically (e.g. if Auer rods are present) but SBB/MPO cytochemistry is necessary to identify the remainder. Non-specific esterase/CE identifies most cases of AML M5 but, in a minority of cases of AML M5a, SBB/MPO and NSE/CE reactions are negative and the diagnosis is based on cytology combined with immunophenotyping. M0 AML is not detected by SBB/MPO and cytochemistry is not generally useful in the

Table 3. Role of cytogenetic analysis

Condition	Diagnostic value	Prognostic value	Affects choice of treatment	Indicated to monitor disease
AML	Yes	Yes	Yes	Yes
ALL/BL	Yes	Yes	Yes	Yes
MDS	Yes	Yes	Yes	Rarely
AA	No	Rarely	Rarely	Not relevant*
CGL	Yes (95%)	Yes	Yes	Yes
MPD	Rarely	Rarely	Rarely	Not relevant
NHL	Yes	Yes, but only in context of histology	No	Yes

* Unless dysplastic features appear.
AML, Acute myeloid leukaemia; ALL/BL acute lymphoblastic leukaemia/Burkitt's lymphoma; MDS, myelodysplastic syndromes; AA, aplastic anaemia; CGL, chronic granulocytic leukaemia; MPD, myeloproliferative disorders; NHL, non-Hodgkin's lymphoma

diagnosis of M7 AML. Sudan black B/MPO may also detect Auer rods in cases of MDS in which none are detectable on a Romanowsky stain. Finally, SBB/MPO and NSE/CE sometimes demonstrate myeloid differentiation in blast transformation of CGL and other MPD.

The Perls' stain for iron is indicated in all cases of suspected MDS since it may provide evidence to support the diagnosis and is necessary for the further categorization of cases of refractory anaemia or refractory anaemia with ring sideroblasts. Occasionally a haemoglobin H preparation is indicated to investigate suspected acquired haemoglobin H disease and a Kleihauer test can be used to demonstrate the presence of cells containing haemoglobin F.

The neutrophil alkaline phosphatase (NAP) and tartrate-resistant acid phosphatase (TRAP) reactions are less useful cytochemical reactions. A low NAP score supports a diagnosis of CGL (Ph-positive or -negative) but is redundant if cytogenetic or molecular genetic analysis is available. The NAP score is normal in about 5% of cases of uncomplicated chronic phase CGL and this can lead to unnecessary uncertainty about the diagnosis in otherwise typical cases. The NAP score can also be reduced in aCML, paroxysmal nocturnal haemoglobinuria (PNH) and 30–50% of cases of MDS. Thus a patient with CMML may have a normal or low NAP score. Tartrate-resistant acid phosphatase positivity is present in the great majority of cases of hairy cell leukaemia and in some cases of the variant form. Positive reactions are, however, also seen in a minority of cases of B-prolymphocytic leukaemia and splenic lymphoma with villous lymphocytes. Whether a laboratory needs to perform NAP and TRAP reactions is therefore dependent on whether they have access to techniques yielding more specific results.

With the advent of immunophenotyping, the periodic acid–Schiff and acid phosphatase reactions are no longer necessary in haematological diagnosis.

Immunophenotyping

Immunophenotyping is essential in the diagnosis of ALL and M0 and M7 AML. It is therefore indicated in all cases of acute leukaemia which are not demonstrated to be myeloid with a Romanowsky stain and cytochemistry. Although immunophenotyping is not yet of importance in determining choice of treatment in AML, the results may indicate groups with poor prognosis, such as those with CD7 positivity (Urbano-Ispizua *et al.* 1992; Kita *et al.* 1993) or CD34 positivity (Geller *et al.* 1990; Solary *et al.* 1992).

Immunophenotyping is useful in determining blast cell lineage in transformation of CGL, since about one-third of transformations are lymphoblastic. (Lymphoid and myeloid transformations cannot be reliably distinguished by cytochemistry since the blast cells in myeloid transformation are often either megakaryoblasts or very undifferentiated myeloblasts, resembling those of M0 AML.) Immunophenotyping is of little importance in investigating transformation of other MPD since transformation is almost always myeloid.

Immunophenotyping is essential in LPD. The demonstration of clonality is important when the diagnosis of a lymphoid neoplasm is in doubt and when it is uncertain if a plasma cell infiltrate is reactive or neoplastic. When there is an established diagnosis of LPD, immunophenotyping is very important in establishing lineage and making a specific diagnosis (BCSH 1994b).

An unusual application of immunophenotyping is its

ability to support a diagnosis of PNH. Immunophenotyping of PB neutrophils and erythrocytes by flow cytometry, to detect reduced expression of CD59 and CD58 respectively, may be more sensitive than Ham's test in detecting the emerging PNH clone in cases of aplastic anaemia (Hillmen *et al.* 1992). (Suitable antibodies, BRIC 5 (CD58) and BRIC 229 (CD59), are available from IBGRL, Blood Products Laboratory, Elstree, Herts, UK).

Cytogenetic analysis

Cytogenetic analysis of the BM should normally be performed at diagnosis in all cases of acute leukaemia. The karyotype is used to stratify patients into good, poor and intermediate prognostic groups (Table 4). The chromosomal abnormality provides a totally leukaemia-specific clonal marker which indicates the choice of chromosomal or breakpoint-specific probes which can be used, with sensitive molecular techniques, to monitor remission. Cytogenetic analysis can also be useful when the diagnosis of AML is in doubt. Since the role of all-trans-retinoic acid in M3 and M3 variant AML has been defined cytogenetic analysis (to detect t(15;17)(q22;q11–12)) has become relevant not only to prognosis but also to the choice of therapy.

Prognostic groups in ALL are shown in Table 4. The worst prognostic groups, Ph-positive cases and those with t(4;11)(q21;23), need the most intensive protocols or BM transplantation. Survival of t(1;19)(q23;p13) patients is greatly improved by intensive therapy (Rivera *et al.* 1991) while hyperdiploidy with 51–68 chromosomes and dic(9;12)(p11;p12) are indications for sparing the patient the more intensive treatment regimes at any age. Because of the more successful outcome with alternative intensive chemotherapy (Murphy *et al.* 1986; Hann *et al.* 1990), the confirmation of Burkitt's lymphoma-related ALL by karyotypic analysis is an important priority.

Karyotypic analysis is important in neonates with apparent congenital leukaemia in order to distinguish transient abnormal myelopoiesis in Down's syndrome (including mosaic Down's syndrome which may not be phenotypically readily apparent) from other cases of congenital leukaemia which are often associated with the very adverse t(4;11)(q21;q23) karyotype. Although it is likely that transient abnormal myelopoiesis in Down's syndrome is actually spontaneously remitting AML this diagnosis does not have

Table 4. The prognostic significance of some cytogenetic abnormalities*

	Prognosis		
Disease	Good	Intermediate	Poor
AML	t(8;21)(q22;q22) t(15;17)(q22;q12) inv(16)(p13q22)	+8 11q23†	t(9;22)(q34;q11) t(6;9)(p23;q34) inv(3)(q21q26) 12p- complex (often including abnormality of chromosome 5, 7 or both)
ALL	Hyperdiploidy with 51–68 chromosomes dic(9;12)(p11;p12)		t(8;14)(q24;q32)‡ t(2;8)(p11.2;q24)‡ t(8;22)(q24;q11)‡ t(9;22)(q34;q11) t(4;11)(q21;q23) t(1;19)(q23;p13)§
MDS	Normal Isolated 5q- Isolated 20q-	+8	−5 −7 7q- inv(3)(q21q26) t(6;9)(p23;q34) complex

*This list is not exhaustive. †Except t(4;11)(q21;q23). ‡Prognosis is greatly improved with alternative chemotherapy (Murphy *et al.* 1985, Hann *et al.* 1990). §Prognosis is greatly improved with intensive chemotherapy (Rivera *et al.* 1991). AML, Acute myeloid leukaemia; ALL, acute lymphoblastic leukaemia; MDS, myelodysplastic syndromes.

the same implications as that of other forms of congenital leukaemia since remission may occur with only supportive management.

Cytogenetic analysis is not recommended for patients with acute leukaemia in remission since it is not sufficiently sensitive to detect low levels of the clone. If monitoring of the leukaemic clone is needed, molecular techniques are required. The karyotype of a relapse BM will distinguish between return of the original leukaemia and induction of a new neoplasm.

In suspected MDS, demonstration of a clonal cytogenetic abnormality confirms the diagnosis, although it must be borne in mind that a normal karyotype does not exclude the diagnosis. Karyotypic abnormalities which in AML are associated with a poor prognosis are, when found in MDS, predictive of early leukaemia transformation or death from complications of cytopenia. The prognostic significance of specific cytogenetic abnormalities is shown in Table 4. It will be noted that in MDS only a normal karyotype and 5q- or 20q- occurring as single abnormalities are indicative of a relatively good prognosis. Karyotype influences choice of therapy, e.g. iron chelation, therapy appropriate for refractory anaemia with 5q- as the only cytogenetic abnormality, would have little relevance in a patient with inv(3)(q21q26) or t(6;9)(p23;q34) in whom early blast transformation and death would be likely. In childhood myelodysplastic/myeloproliferative disorders, cytogenetic analysis will help to distinguish the infantile monosomy 7 syndrome from juvenile chronic myeloid leukaemia.

In aplastic anaemia, cytogenetic analysis is not indicated if resources are short. It is rarely successful and the detection of a clonal cytogenetic abnormality is rare. Nevertheless, a chromosomal abnormality found in an otherwise typical aplastic anaemia is indicative of a neoplastic clone and is of importance for prognosis and choice of therapy. The development of dysplastic features in aplastic anaemia suggests that a neoplastic clone is present and is thus an indication for cytogenetic analysis.

Among chronic myeloid leukaemias, it is important to identify the Ph-positive cases including those with variant translocations. Although there is little evidence that the variants differ from the classical 9;22 translocation in their prognostic significance their detection provides an important baseline from which to monitor the course of the disease. The presence of additional karyotypic abnormalities at diagnosis or their development during the course of the illness are indicative of clonal evolution and may herald an accelerated phase or blast transformation. Cytogenetic response to treatment (interferon or BM transplantation) is a valuable and prognostically important investigation in CGL. However, DNA or RNA analysis, to detect the BCR-ABL chimeric gene, are more sensitive in monitoring the leukaemic clone and in Ph-negative CGL they replace cytogenetic analysis for this purpose.

Cytogenetic analysis is not generally useful in other MPD. However, in patients presenting as essential thrombocythaemia, it is important to detect the poor risk groups, e.g. Ph-positive cases and those with inv(3)(q21q26). Cytogenetic analysis is therefore indicated in patients with atypical features, e.g. marked basophilia or blast cells in the PB.

In LPD, cytogenetic analysis may confirm a provisional diagnosis, e.g. when t(14;18)(q32;q11) is detected in suspected follicular lymphoma, t(11;14)(q13;q32) in suspected mantle cell lymphoma or t(2;5)(p23;q35) in suspected T-lineage anaplastic large cell lymphoma. However, most translocations are not totally specific for a histological category so karyotype must be interpreted in the light of the cytological features. t(11;14), for example, is detected not only in mantle cell lymphoma but also in a significant proportion of patients with splenic lymphoma with villous lymphocytes and in some patients with B-prolymphocytic leukaemia. Cytogenetic analysis can be important in demonstrating clonality and thus confirming a diagnosis of large granular lymphocyte leukaemia, particularly in CD3-negative cases in which there is no rearrangement of T-cell receptor genes. In LPD, cytogenetic abnormalities have prognostic significance, but only when the precise diagnosis and the karyotype are considered together. For example, t(14;18) when detected in a large cell lymphoma does not have the same significance as when detected in a centroblastic/centrocytic lymphoma. In general, when resources are scarce, cytogenetic analysis is not indicated in lymphoma unless it is likely to give important prognostic information or influence choice of therapy.

The role of cytogenetic analysis in haematological neoplasms is summarized in Table 3.

Practical points

When blood samples for immunophenotyping and cytogenetic analysis are sent to specialist centres, certain procedures must be followed if optimal results are to be achieved and waste of valuable resources is to be avoided. All samples should be accompanied by full clinical details, the provisional diagnosis, the results of a full blood count and, in the case of immunophenotyping, a film of PB, BM or both. It may be necessary to despatch samples to the specialist centre before full information is available but in this eventuality a follow-up telephone call is essential. It is important to stress, for example, that, in LPD, immunophenotyping and karyotypic analysis of the BM are only relevant when there is infiltration by neoplastic cells. A

BM film should be examined promptly and, if there is no infiltration, the specialist laboratories should be notified immediately so that processing of the sample does not proceed. This is particularly important for cytogenetics laboratories where most of the labour-intensive work remains to be done.

For immunophenotyping, heparinized or EDTA-anticoagulated samples are suitable. Samples should reach the referral laboratory within 24 h. They should not be refrigerated and should be transported at ambient temperature. Either PB or BM is suitable, as long as neoplastic cells are present. Even samples containing a low proportion of neoplastic cells may give useful information. However, if the percentage of neoplastic cells is low a larger volume of blood should be sent, e.g. 40–50 ml, whereas if the count is very high 8–12 ml will suffice.

Samples for cytogenetic analysis should be taken into 5 ml of tissue culture medium containing 100 IU units of preservative-free heparin. Samples should not be refrigerated and should be transported at ambient temperature to reach the specialist laboratory within the same working day. Either PB or BM may be suitable. For acute leukaemia, a BM specimen is preferred but PB is an alternative if the white cell count is high (e.g. $>20\times10^9$/l) and if the blast count is significant (e.g. $>30\%$). In CGL, BM is preferred but, in cases with a high white cell count in whom no BM specimen is available, PB may be satisfactory. In MDS a BM specimen is needed.

Conclusion

A precise diagnosis is necessary in haematological neoplasms in order to determine prognosis and guide the choice of treatment. A specific diagnosis always requires cytology and often also requires cytochemistry, immunophenotyping and cytogenetic analysis. The relative importance of each of these methods of investigation varies according to the differential diagnosis in an individual patient. Whether specific investigations are required in an individual laboratory depends on the frequency with which patients with haematological neoplasms are encountered and on the ease of access to regional or supra-regional diagnostic services.

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