

Leader

Immunophenotyping in the diagnosis of acute leukaemias

General Haematology Task Force of BCSH

Introduction

Immunophenotypic analysis of the reactivity of leukaemic cells with monoclonal antibodies has proved useful and nowadays essential in the diagnosis of acute leukaemias. This was first shown to be relevant in the characterisation and classification of acute lymphoblastic leukaemias of various cell types^{1,2} as there is no specific and reliable cytochemical marker to recognise lymphoblasts. Subsequently, immunological markers have also been shown to be important in the diagnosis of acute myeloblastic leukaemias, particularly when the nature of the blasts cannot be defined by morphology and cytochemistry. Examples of these "undifferentiated" acute leukaemias include those with poorly differentiated myeloblasts (AML-M0),³⁻⁵ or those derived from early erythroid and megakaryocyte precursors.⁶

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There is currently a large panel of monoclonal antibodies (over 1000) available which detect different molecules (over 70) on normal haemopoietic and leukaemic cells of various lineages. These monoclonal antibodies are grouped according to the molecule or antigen that they recognise under a cluster designation or differentiation (CD) number which extends from 1 to 76. Only a minority of these reagents have been shown to be helpful for the diagnosis of acute leukaemia: these comprise monoclonal antibodies which recognise antigens in lymphoid/myeloid cells from the earlier stages of differentiation and which, with few exceptions, are cell lineage restricted. In this review an outline will be given of:

- (i) The panel of monoclonal antibodies which are of practical use in the diagnosis of acute leukaemia.
- (ii) The advantages and disadvantages of the different techniques which can be applied to detect the cell reactivity with a monoclonal antibody.
- (iii) A number of considerations or recommendations related to the methodology and interpretation of the findings.
- (iv) a detailed description of the methodologies for immunophenotyping.
- (v) Selection of reagents.
- (vi) Indications for immunophenotyping.

Panel of monoclonal antibodies for the diagnosis of acute leukaemia

The most useful reagents to characterise and distinguish B and T cell derived acute lymphoblastic leukaemias (ALL) and acute myeloid leukaemias (AML) are illustrated in figure 1. Thus a first line panel of monoclonal antibodies will include 10 reagents: three B cell associated markers: CD19, CD22 (cytoplasmic), CD10; three T cell associated markers: CD7, CD3 (cytoplasmic), CD2; three myeloid associated markers: CD33, CD13 (cytoplasmic), anti-myeloperoxidase (anti-MPO), and an antibody that detects the nuclear enzyme terminal deoxynucleotidyl transferase (TdT).

All types of B lineage ALL (TdT+, CD19+, CD22(cyt)+, CD10+/-), all T lineage ALL (TdT+, CD7+, CD3(cyt)+, CD2+/-), and AML (CD33+/-, CD13(cyt)+/-, anti-MPO+/-) can be detected, using these 10 reagents.^{2,5,7,8}

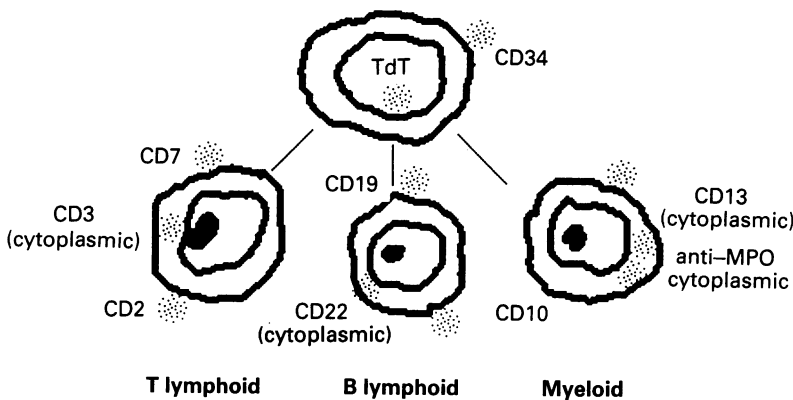


Figure 1 Monoclonal antibodies for the characterisation of acute lymphoblastic and myeloid leukaemias.

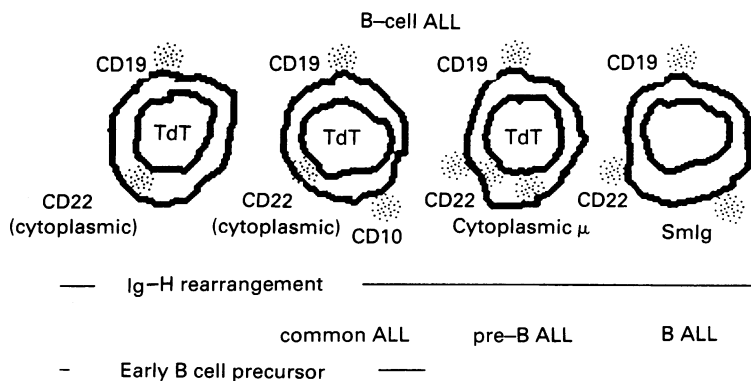


Figure 2 Immunological classification of B lineage acute lymphoblastic leukaemias.

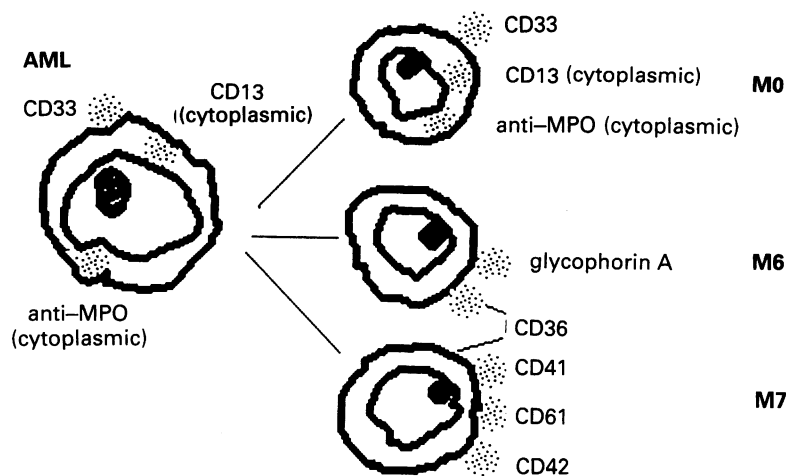


Figure 3 Monoclonal antibodies for the characterisation of the various subtypes of acute myeloid leukaemia.

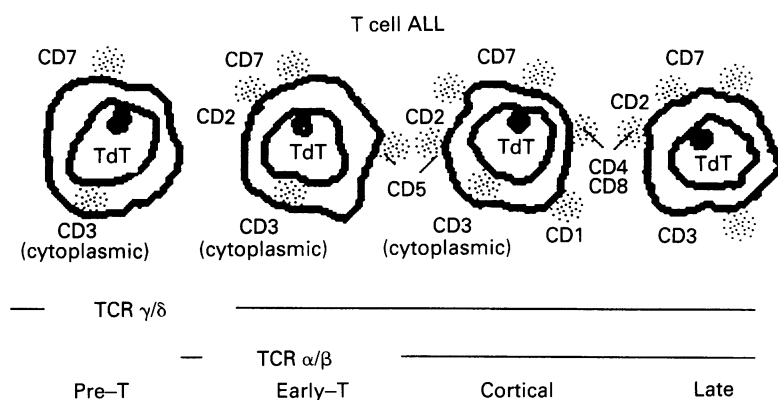


Figure 4 Immunological classification of T lineage acute lymphoblastic leukaemias.

A second panel of monoclonal antibodies can be used to dissect further the various ALL and AML subtypes, but these are not essential and are only of value in particular cases:

(1) Cytoplasmic μ chain (cyt μ) and surface immunoglobulins (Smlg) to subclassify further the various B lineage ALL into: early B lineage ALL (CD19+, cyt CD22+), common ALL (CD10+), pre-B ALL (cyt μ +), and B ALL (Smlg+) (fig 2).

(2) CD41 (anti-platelet glycoprotein IIb/IIIa) or CD61 (anti-platelet glycoprotein IIIa) to detect megakaryoblastic (M7)-AML and the monoclonal antibody anti-glycophorin A for the diagnosis of erythroleukaemias (fig 3). These may be of great value for the diagnosis of some acute "undifferentiated" leukaemias, when all the markers used in the screening panel are negative.

(3) Other T associated markers (CD5, CD1, CD4, CD8) are recommended to characterise further T lineage ALL (fig 4).

Optional markers which may add some information, though they are not essential for diagnostic purposes because of the lack of specificity are: CD34 (stem cell associated), anti-class-II HLA-Dr determinants, and CD14.

Although most acute leukaemias can be classified with such a panel of reagents as lymphoid or myeloid, a few acute leukaemias, designated biphenotypic or mixed lineage, in

which the blasts express a constellation of myeloid and lymphoid antigens, cannot. The nature of the leukaemic cells in these cases is unknown but they probably represent malignancies of stem cells.^{9,10}

Methodology

Essentially there are two types of techniques which can be applied for routine immunophenotyping: (i) immunofluorescence on viable, unfixed cells in suspension; (ii) immunoenzymatic methods—immunoperoxidase and immunoalkaline phosphatase (APAAP) on fixed cells on slides.

Both techniques are reliable. The use of one or the other, or both, will depend on the facilities in each laboratory; however, the immunoenzymatic methods are recommended for a district general hospital.

The advantages and disadvantages of the two methods are summarised in the table.

Recommendations

The laboratory wishing to embark on immunophenotyping has to take into account a number of considerations:

- (1) Before setting up the technique, the laboratory worker should spend at least one week in a unit with experience of immunophenotyping.
- (2) Specimens from healthy individuals should be tested before analysing leukaemia specimens.
- (3) Results must be interpreted in the context of the specimen being analysed, particularly in those with a mixture of leukaemic and normal cells.
- (4) Results should be interpreted with knowledge of the cytological features. Lack of specificity must always be considered.

For example, reactivity with CD19 and

Advantages and disadvantages of two immunological methods applied on cell suspensions and fixed cells in smears

Immunofluorescence (cell suspension)	Immunoenzymatic (slides-fixed cells)
Advantages	
Preservation of antigens as cells are unfixed.	Permits retrospective studies
Quantitation of antigenic sites	Detection of membrane and cytoplasmic antigen
Analysis of large number of the cells (flow cytometry)	Allows identification of the morphology of positive cells
Permits double labelling on a routine basis	Results can be reviewed as slides are kept
	Requires only ordinary light microscope
Disadvantages	
Requires a fluorescence microscope or flow cytometer	Some antigens may be destroyed by the fixative
No detection of cytoplasmic antigens with standard method	No discrimination between cytoplasmic and membrane staining
Retrospective studies are not possible	Quantitative measurement of antigenic sites is not possible
Difficulties in interpretation of data in heterogeneous samples or those with few blasts	

CD22, when no assessment of TdT has been made, may correspond to a B cell lymphoma as well as to ALL. The specificity of the individual markers needs also to be considered—for example, the monoclonal antibody CD7 on its own does not identify as T lineage ALL a particular blast population because about 20% of AML cases are CD7 positive; in addition, a proportion (about 20%) of AML cases are also TdT positive.

(5) There is no consensus on the cutoff point which indicates that a sample is positive for a marker. The following are recommended as cutoff points: 20% blast cells positive with a given marker when samples are analysed by flow cytometry, and 10% when assessed by immunocytochemistry.

(6) Some monoclonal antibodies—CD3, CD22, CD13—are useful reagents when reactivity is detected in the cytoplasm of the cells as they are expressed first in the cytoplasm and later in the membrane.²⁷⁸ Therefore, these markers should be assessed on fixed cells. This is also true for anti-MPO which is detected only in the cytoplasm and not in the cell membrane.

(7) Blood and bone marrow samples must be collected in anticoagulant. Preservative-free heparin is a suitable anticoagulant. Such specimens are also suitable for cytogenetic studies and other laboratory tests. Nevertheless, K₂ EDTA and K₃ EDTA are also satisfactory for cell marker studies.

(8) Specimens ideally must be processed as soon as possible after collection. However, they may be stored for 24 hours either at room temperature (whole blood) or at 4°C (isolated mononuclear cells) before immunophenotyping. When the specimens are not fresh (over 24 hours), a viability test should be performed to exclude false binding by non-viable cells. Addition of tissue culture medium (such as RPMI-1640) is recommended when the specimens are likely to be stored beyond 24 hours. For immunoenzymatic techniques, slides should be dried at room temperature for 24 to 48 hours. Thereafter, if there is any further delay in carrying out the test, slides should be kept unfixed at -20°C wrapped in foil or polyvinyl film paper until the test is performed. The choice of fixative will depend whether expression of cytoplasmic or nuclear antigens is being studied. For instance, cold acetone for 10 minutes is a suitable fixative for the detection of both cytoplasmic and nuclear antigens while cold methanol is the best fixative for the detection of nuclear antigens.

(9) Dilutions of the monoclonal antibody and the second layer reagents should be carefully set up in each laboratory to avoid false positive or negative results. In this context positive and negative controls should always be used. This is absolutely essential when a new batch of reagents is introduced.

Methodology for immunophenotyping

Peripheral blood and bone marrow samples are collected in anticoagulant and mononu-

clear cells are isolated by Ficoll density centrifugation and washed twice in phosphate buffered saline (PBS) with 1% bovine serum albumin (Sigma), 0.05% sodium azide, and 2% human AB serum (pH:7.6) (PBS-BSA-azide-AB buffer). Cells from pleural fluid, ascitic fluid and cerebrospinal fluid are resuspended also in the same buffer, which is used in all washes.

IMMUNOFLUORESCENCE

For the detection of cell surface antigens by indirect immunofluorescence, 50 µl of a cell suspension (10⁶ cells) is incubated with the adequate volume (ranging from 5 µl to 50 µl) of the optimally diluted monoclonal antibody for 30 minutes at 4°C. After two washes with the buffer PBS-BSA-azide-AB, the cells are incubated with the fluorescein conjugated (FITC) anti-mouse immunoglobulin F(ab)₂ fragment (optimally diluted) for 30 minutes at 4°C, washed again twice in the buffer, and the cell pellet resuspended in glycerol/PBS (1/1), mounted on a glass slide, covered with a coverslip and sealed to be evaluated under the fluorescence microscope. Addition of anti-fade reagent, such as 1,4-diazabicyclo-(2.2.2)-octane, to the mounting medium glycerol/PBS may be useful to facilitate the reading under the fluorescence microscope. Phase contrast microscope is recommended and at least 200 cells should be analysed for the fluorescence stain; a positive cell is seen with multiple fluorescence dots around the membrane.

The same procedure is applied for flow cytometry analysis, but following the incubation with the FITC anti-mouse immunoglobulin and subsequent washes, the cell pellet is resuspended in 500 µl of Isoton and assessed on the flow cytometer. If the sample is to be analysed more than four hours after staining, the cell pellet should be fixed in 1% paraformaldehyde in 0.85% saline instead of being resuspended in Isoton.

Whole blood can be also tested by flow cytometry without the need for isolation of the mononuclear fraction according to the manufacturer's instructions. Usually, 100–200 µl of blood are incubated with the monoclonal antibody, followed by the addition of FITC conjugated antimouse immunoglobulin. The methodology is identical to that described above but the buffer used does not contain human serum and the pellet, following the immunostaining, is treated with 1 ml of lysing solution for 10 minutes at room temperature and washed twice in buffer (PBS-azide). Finally, the cell pellet is resuspended in 250 µl of Isoton and the measurement is performed in the flow cytometer.

In some circumstances, for instance in biphenotypic/mixed lineage acute leukaemias, or for the detection of minimal residual disease, it may be useful to apply a double labelling technique to determine whether a single cell population coexpresses two different antigens in the membrane. Usually, a direct immunofluorescence technique using two directly conjugated monoclonal antibod-

ies to two fluorochromes (such as fluorescein and phycoerythrin) is applied. The monoclonal antibodies should be independently titrated and the methodology is similar to that of the indirect immunofluorescence described above with the omission of the second incubation step.

Negative control preparations should be set up by replacing the relevant monoclonal antibody with a mouse immunoglobulin of the same isotype, and when possible positive controls should be performed using normal or leukaemic blood samples. For practical purposes, a mixture of mouse immunoglobulins of the various isotypes can be used as the negative control.

IMMUNOALKALINE PHOSPHATASE (APAAP)

This may be carried out on smears or on a layer of mononuclear cells prepared on a cyto-centrifuge. Both should be air dried for at least six hours before proceeding with the test. The location of the cells is marked by encircling the area with a glass pencil. Fixation is done in cold pure acetone for 10 minutes at room temperature. A water repellent (Sigmacote) is applied around the circle and the slides are placed in a humid chamber and incubated with 50 μ l of optimally diluted monoclonal antibody for 30 minutes. From now on the cells must not be permitted to dry. Following a wash with 0.05 molar TRIS-buffered saline (TBS) (pH 7.6), the slides are incubated with 50 μ l of a rabbit antimouse immunoglobulin diluted 1 in 20 in TBS and 2% human serum for 30 minutes, washed again in TBS, and incubated for 45 minutes in the immunoalkaline phosphatase anti-alkaline phosphatase (APAAP) complexes diluted 1 in 60 in TBS. Following a wash in TBS the spread cells are incubated for 20 minutes in a previously filtered developing solution which contains Naphthol AS-Mx phosphate, Levamisole, N,N-dimethylformamide and Fast Red TR salt, rinsed in distilled water, and counterstained with Harris's haematoxylin for 10 to 20 seconds. The slides are mounted with Glycergel (Dako) without allowing them to dry and assessed under an ordinary light microscope. To assess the nuclear enzyme TdT, an extra incubation step is required following the fixation and initial incubation with the rabbit anti-TdT antibody. This requires incubating the cells for 30 minutes with a mouse anti-rabbit immunoglobulin before the incubations with the rabbit anti-mouse immunoglobulin and the APAAP complexes as outlined above.

Negative controls are set up by replacing the monoclonal antibody with a mouse immunoglobulin of the same isotype, and smears from normal blood and bone marrow are used as positive controls when required.

Selection of reagents

Different antibodies with the same CD number are not always equally satisfactory as diagnostic reagents. Reagents should be selected on the basis of their well established speci-

ficity, stability, and potential for use in detecting membrane and cytoplasmic antigens and also their cost which is generally high. Polyclonal antibodies are preferable for the detection of immunoglobulin light chains (κ and λ).

It is recommended that before reagents are selected advice is sought from laboratories experienced in immunophenotyping. The laboratory worker should also assess the reliability of the reagents by analysing samples known to be positive and negative for a particular marker.

Indications for immunophenotyping

Immunophenotypic analysis should be systematically performed in all cases of acute leukaemia and blast transformation of chronic myeloid leukaemia or other myeloproliferative disorders (such as myelofibrosis) and myelodysplasia.

This analysis is mandatory when the leukaemic blasts are morphologically undifferentiated and negative for the cytochemical reactions characteristic of myeloid cells: Sudan black B and myeloperoxidase for the granulocytic line; and non-specific esterases for the monocytic line. In these cases immunophenotypic analysis may clarify whether the case corresponds to one of the subtypes of ALL or to poorly differentiated acute myeloid leukaemias (AML-M0), early erythroleukaemias, or megakaryoblastic leukaemias.

In the remaining cases of acute leukaemia in which the blasts are shown to be myeloid by standard light microscopy, morphology, and cytochemistry (such as presence of Auer rods or myeloperoxidase activity), the immunological markers are not essential as in the group outlined above. Still, it is recommended that the immunological profile of these blast cells be investigated to rule out cases of biphenotypic/mixed lineage acute leukaemia^{9,10} in which the blasts have myeloid and lymphoid features despite presenting as AML.^{9,10}

Immunological markers are not useful in cases of chronic myeloproliferative disorders and myelodysplasia with no evidence of blast transformation.

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