
Materials and Methods

Immunophenotyping by Flow Cytometry: Leukaemia Panels

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Introduction

The immunological characterization of surface antigens is a major tool for the study and classification of leukaemias. Flow cytometry is the methodology used to detect cell surface antigens using monoclonal antibodies conjugated with different fluorochromes. This laser-based instrumentation has replaced the use of the fluorescence microscope for the detection of surface antigens.

Immunophenotyping is the investigation of the presence of specific antigens on the surface, the cytoplasm or the nucleus of a cell. In clinical practice, immunophenotyping of leukaemia samples is used to characterize the leukaemic cell, to follow up treatment by the detection of minimal residual disease, and to identify and isolate CD34+ stem cells from the peripheral blood for treatment purposes.

The value of the characterization of leukaemic cells is:

- To establish the lineage for the predominant cell type (i.e. myeloid, T-lymphoid or B-lymphoid)
- To establish the stage of maturity of the predominant cell type (i.e. blast cells in acute leukaemia or mature cell immunophenotype in B-chronic lymphoproliferative disorders)
- To demonstrate clonality in the B-lymphoid malignancies by detecting either kappa or lambda light chain restriction
- To establish dual lineage in biphenotypic leukaemias
- To detect rare immunophenotypic profiles which characterize certain disease entities
- To demonstrate unusual co-expression of antigens or aberrant profiles which typify their disease in certain patients and can be used to monitor minimal residual disease after treatment

In order to obtain the information discussed above in a cost-effective manner, there is a set group of different monoclonal antibodies selected specifically to use for different diseases; this is called a panel. The chosen panel will show a particular immunophenotypic profile that is of diagnostic use because it is characteristic of some diseases. The use of the wrong panel, or one that is too small or restricted, often fails to define phenotype sufficiently well or may miss an important differential diagnosis.

The implementation of leukaemia panels is frequently discussed. However, there is no consensus on the selection of the McAb panel for the general laborato-

ry involved in immunophenotyping. There have been several worldwide attempts to recommend guidelines for panels to diagnose acute and chronic leukaemia and these reflect the different approaches in several parts of the world. In the UK, the General Haematology Task Force of the BCSH published the latest recommendations for immunophenotyping of acute leukaemia and chronic lymphoproliferative disorders as far back as 1994; an update is being prepared and will be published soon. The European Group for the Immunological Characterisation of Leukaemias (EGIL) published their proposals for the immunological classification of acute leukaemias in 1995. This work is based on the group's recommendations.

In practice, laboratories select their leukaemia panel according to their workload and the characteristics of the samples that are most frequently processed. Another consideration affecting the selection of a panel is the cost of the monoclonal antibodies. All these variations make it difficult to implement a panel suitable for all the laboratories in the UK.

Methods

Recent important technical advances in the area of immunophenotyping, and new reagents and methodology have become available; for instance:

- The detection of cytoplasmic and nuclear antigens with simultaneous staining of membrane antigens by flow cytometry
- The use of double and triple staining with McAb directly conjugated
- The analysis approach of looking at pattern of expression as well as the percentage of positivity
- The use of new techniques and reagents for whole blood lyse-no-wash
- New gating strategies with the use of CD45, back gating and sequential gating

Detection of intracellular antigens

Cytoplasmic and nuclear antigens can easily be detected by flow cytometry using a variety of commercially available permeabilisation and fixation solutions. Not all commercially available solutions are equally reliable for detecting intracellular antigens. It is also possible to stain simultaneously for membrane and cytoplasmic or nuclear antigens. This is of particular application in the differentiation of normal B-cell precursors from minimal residual disease in acute lymphocytic leukaemia.

Use of triple staining

Triple staining is used as a routine method, mainly for HIV typing, in leukaemia. It also provides valuable information for the detection of kappa and lambda combined with CD19 third colour. It is essential when performing CD45 gating.

Whole blood lyse-no-wash method

This methodology is frequently recommended for instances where there are rare events to be identified, because it minimises the loss of cells in samples at high risk of infection. This technique requires McAb designed specifically for this use.

Pattern recognition

More informative data are obtained by looking at the intensity of antigen expression as well as the percentage of cells that are positive for that particular McAb. This has been shown in the differentiation of T-cells, in the expression of CD10 and TdT in acute leukaemia, in the intensity of immunoglobulin light chain expression in B-cell chronic lymphoproliferative disorders, in CD34 expression in acute myeloid leukaemias, etc.

Gating strategies

The traditional SSC /FSC gating for leukaemia is still appropriate and used widely. However, when detecting rare events, CD45 gating is more accurate to gate on leukocytes only, and further sequential gating is essential in those cases. Other options are: back gating on an expression of a specific antibody, using side scatter and the fluorescence channel and also gating using a modified side scatter.

Discussion of immunophenotyping panels

1. Stem cell (CD34) panel

Stem cells characterised by the expression of CD34 are capable of reconstituting long term multilineage haematopoiesis after ablative therapy. Transplant centres routinely rely on CD34+ quantitation by flow cytometry, as markers of pluripotential stem cells to determine the optimal timing and to confirm the adequacy of haematopoietic progenitor cell harvests. A minimum threshold dose between 2 and 5 × 10⁶ CD34+ cells/kg bodyweight has been demonstrated to be adequate for engraftment. Although expressed in haematopoietic progenitors, CD34 is not restricted to them; it is also found in endothelial cells and some stromal cells. CD34 is localised in chromosome 1, band q32. There are different classes of CD34 antibodies: the CD34 monoclonal antibodies are classified according to their sensitivity to neuraminidase and glycoprotease. Epitopes recognised by class I are sensitive to both enzymes, class II antibodies are sensitive to glycoprotease only and class III are insensitive to both enzymes.

Class I MY10, 12.8, Immuno133, Immuno409, 43.A1

Class II QBEND10, ICH3

Class III Tuk3, HPCA-2, BIRMA-K3

Only class II and III antibodies PE conjugated should be used for CD34+ stem cell enumeration using sequential CD45 gating as described in the ISHAGE protocol; at least 100 CD34+ events or 75.000CD45+ events should be acquired. It is essential to monitor the flow cytometer performance using calibration beads and it is also essential to participate in external quality control schemes.

2. Chronic leukaemia panel

B-cells were originally recognised as the lymphocytes bearing membrane immunoglobulins. They produce antibodies in the form of immunoglobulins as part of their function in the humoral immunological response. The most common B-cell chronic lymphoproliferative disease in the UK is B-CLL, followed by B-NHL; all the other diseases are rarer and are not encountered very often in general practice. These are prolymphocytic leukaemia (B-PLL), hairy cell leukaemia (HCL) and its variant (HCL variant), splenic lymphoma with villous lymphocytes (SLVL) and multiple myeloma. It makes sense to use a primary panel to diagnose B-CLL and B-NHL; if such a panel does not provide a clear diagnosis then it is necessary to add a secondary panel to identify rarer disorders.

Primary panel for the diagnosis of B-CLL based in the scoring system for B-cell chronic lymphoproliferative disorders:

T-cell markers	CD2
B-cell markers	CD23 FMC7 CD79b CD22
	Surface kappa/lambda
B and T-cell marker	CD5

Other optional markers. The combination of CD5/CD19 is of particular importance to demonstrate co-expression of CD5 and B-cell marker (CD19). Nowadays it is necessary to include CD20 in the panel, because some B-cell disorders are treated with an anti-CD20 antibody, and it is essential to know if the CD20 molecule is expressed on the surface of the B-cell lymphocytes. The diagnosis of certain types of lymphoma may be helped by the addition of CD10 to the primary panel. The inclusion of the T-cell markers CD4 and CD8 in the primary panel may be considered for prompt identification of T-cell lymphocytosis and T-cell disorders.

Problems with kappa/lambda detection. The detection of light chain restriction immunoglobulin is a common problem for most laboratories. There are different options and different antibody combinations to detect kappa/lambda; in B-CLL it is preferable to use a polyclonal antibody because the expression of the light chains is extremely weak. The most popular and efficient method is the use of a triple colour combination with CD19 third colour combined with kappa FITC and lambda PE. Inexperienced users of this method may find it difficult to calibrate adequately

the instrument compensation for the three-colour combination. Two-colour admixture with CD19 PE and kappa FITC and CD19 PE and lambda FITC are also common practice.

The detection of light chains in whole blood is more problematic than using gradient separated mononuclear layer. If the red cell lysis is performed after staining, the PB should be washed two or three times in PBS or Hanks before staining to wash out the serum Ig that may block the detection of the light chains. Alternatively, the whole blood could be lysed and washed twice prior to staining.

Secondary panel. If after the implementation of the primary panels the diagnosis is not clear, the use of a secondary panel is indicated for the diagnosis of B-cell diseases other than B-CLL or B-NHL.

Other B-cell markers: CD25 CD38 CD11c CD138 CD103 cytoplasmic kappa lambda. The combination CD11c, CD25, CD38 and CD103 is helpful to identify HCL from other 'hairy'-looking disorders, such as SLVL and HCL variant.

The combination CD38 and CD138 is used for the identification of plasma cells associated with the detection of cytoplasmic kappa lambda.

3. T-cell leukaemia panel

T-cells were identified in 1968 by the observation that they were lymphocytes able to form rosettes with sheep erythrocytes. This panel is recommended in the case where the T-cell markers are positive in the absence of clonal expression of B-cell markers. The objective of this panel is to characterise the maturity of the T-cells in the specimen. The primary differential diagnosis is to demonstrate the presence of a T-cell lymphocytosis of reactive type or one of leukaemic origin. The diagnosis of T-cell disorder should be suspected by cell markers and supported by other tests such as DNA analysis of the TCR, etc.

T-cell chronic leukaemia panel.

Pan T-cell markers:	CD2 CD3 CD5 CD7	TCR α/β TCR
Helper/inducer:	CD4	
Suppressor/Cytotoxic:	CD8	
Natural killer:	CD11b	CD16 CD56 CD57
Activated T-cells:	CD25 CD38 Hla Dr	

In T-cell leukaemias, one of the subsets of CD4+ T-cells or CD8+ T-cells is usually expanded and some of the pan T-cell marker expression may be missing or expressed very weakly. For instance, in Sezary Syndrome, a T-cell cutaneous lymphoma, the T-cells characteristically express CD4 and do not usually express CD7. LGL are characterised by the expansion of the CD8+ T-lymphocytes. In T-PLL the proliferating cell has a mature T-cell immunophenotype; they express CD4 or CD8 and have a strong density of sur-

face CD7.

4. Acute leukaemia panel

Acute leukaemia is the malignant clonal proliferation of haematopoietic cells with a sudden presentation. In the majority of cases, the leukaemic cell is arrested at a normal maturation stage and displays a physiological immunophenotype of an immature cell in its process of differentiation. The objective of selecting a monoclonal antibody panel to classify acute leukaemia in the first instance is to distinguish between the lymphoid and the myeloid forms of acute leukaemia and then, within the former, B-lymphoid from T-lymphoid. The panel is also used to detect possible lineage infidelities and biphenotypic leukaemia.

Primary panel.

B-cell markers:	membrane CD19 CD10 cytoplasmic CD22 CD79a
T-cell markers:	membrane CD2 CD7 cytoplasmic CD3
Myeloid markers:	membrane CD13 CD33 CD117 CD14 CD15 cytoplasmic MPO
Precursor markers:	membrane CD34 Hla Dr nuclear TdT

Secondary panel.

IgM (cyt) and Ig light chains if B-cell phenotype
CD1a, CD3 (membrane), CD4, CD5, CD8 if T-lymphoid phenotype
CD41, CD42 and CD61 (megakaryoblastic leukaemia)
anti-glycophorin A (erythroid leukaemia)

Optional markers.

Anti-lysozyme. This is a monoclonal antibody that detects lysozyme in the cytoplasm and although it is not specific for monoblastic leukaemia it is preferentially expressed in this subtype and it is more sensitive than CD14. The weak expression of CD4 is found in monocytes.

CD15. This is characteristically positive in the subset of pro-B-ALL (CD79a+, CD19+, CD22+, CD10-, cytoplasmic IgM-) with 11q23 rearrangement and therefore its expression may be indicative of such chromosomal abnormality.

B-cell acute lymphocytic leukaemia.

This is characterised by the expression of at least two of the B-cell antigens CD19, CD79a or cyt.CD22 with lack of expression of T and myeloid cell markers.

There are four types of B-cell All (TdT+, CD19+, CD79a+ CD22+) that can be distinguished by markers:

1. Null ALL, early B-cell precursor ALL, pro B-ALL or BI : (CD10-)

2. Common ALL or BII : (CD10+)
3. Pre B-All or BIII: (cytoplasmic IgM+)
4. Mature B-cell ALL or BIV: (surface Ig +)

T-cell acute lymphocytic leukaemia.

This is sub-classified into four groups based on normal thymic differentiation:

1. T-ALL or TI: (CD7+ cyt CD3+)
2. Pre-T-ALL or TII: (CD2+ or CD5+ or CD8+)
3. Cortical T-ALL or TIII: (CD1a+)
4. Mature T-ALL or TIV: (membrane CD3+)

There is an early T-cell leukaemia called Pro-T-ALL, which is characterised by the expression of CD7+ and CD117+ and negative for CD3, CD4 and CD8.

Acute myeloid leukaemia.

This is defined by markers by the expression of two or more myeloid associated markers in the absence of specific lymphoid markers, i.e. MPO CD117 CD13 and/or CD33. The cell marker characterisation of AML does not permit one to distinguish between the FAB classification sub-groups since the latter is a morphological and cytochemical classification.

Anti-lysozyme and CD14 are monocytic markers and are expected to be positive in AML with a monocytic component such as M4 and M5, alongside the myeloid markers CD13 and CD33.

Negative Hla Dr is associated with M3 or late M2 AML. M3 is also associated with co-expression of CD2 and M2 with co-expression of CD19.

Biphenotypic acute leukaemia (BAL).

There is a lack of agreement to define BAL. Below is a reviewed scoring system to facilitate its diagnosis. BAL is defined when the scores are over 2 for the myeloid lineage and 1 for the lymphoid lineage.

B-lineage scoring:

- 2 points for CD79a cyt IgM cytCD22
- 1 point for CD19 CD10 CD20
- 0.5 point for TdT CD24

T-lineage scoring:

- 2 points for cyt CD3 TCR ?? TCR ??
- 1 point for CD2 CD5 CD8 CD10
- 4.5 points for TdT CD7 CD1a

Myeloid lineage scoring

- 2 points for MPO
- 1 point for CD117 CD13 CD33 CD65s
- 4.6 points for CD14 CD15 CD64

5. Minimal residual disease panel

With the advances in treatment for acute leukaemia, the detection of minimal residual disease is increasingly requested by clinicians. So far there is not a reliable

panel of antibodies to detect MRD in acute myeloid leukaemia, but the combination of TdT with CD10 and TdT with CD19 can differentiate between normal regenerating B-cell precursors and MRD in ALL by their pattern of expression. Normal B cell precursors show a pattern of brighter TdT expression and dimmer CD10 than the leukaemic B-cells. All CD19+ leukaemic B-cells are TdT positive and only 50% of the TdT+ normal B-cell precursors co-express TdT and CD19.

Conclusions

There is a need for a consensus in the election of leukaemia panels. In general, the majority of laboratories perform a panel adapted to their own practice. This situation is acceptable, provided that the panel is flexible and adaptable to different situations encountered in day-to-day practice. The most important considerations are the right interpretation of the results for the chosen panel and ensuring that the reports of those results are given in a meaningful manner to the clinician.

The participation in an internal and external quality control scheme to monitor the performance of the laboratory and the people involved in the diagnosis of specimens sent for immunophenotyping is another essential part of good laboratory practice.

Further reading

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