

Leaders

Immunophenotyping in the diagnosis of chronic lymphoproliferative disorders

General Haematology Task Force of BCSH

Introduction

As in the acute leukaemias, immunophenotyping has been shown to be useful in the characterisation and classification of the chronic lymphoproliferative disorders.^{1,2} This term refers to a group of diseases caused by the clonal proliferation of B and T lymphocytes. Recognition of the various disease entities is essential because of major implications for prognosis and patient management.

The diagnosis of the lymphoproliferative disorders requires use of multiple technologies including immunological markers, cell morphology and, in some cases, histopathology of the affected tissues and molecular and cytogenetic investigations.² Immunophenotyping is a key laboratory test in the precise diagnosis of these conditions.

This article discusses the role of immunological markers in the diagnosis and characterisation of the lymphoproliferative disorders and provides guidance on: (1) methodological aspects; (2) panels of markers useful for diagnostic purposes; (3) interpretation of the results and; (4) indications for immunophenotyping.

Role of immunological markers in the characterisation of lymphoproliferative disorders

The value of immunological marker studies as a diagnostic tool in chronic lymphoproliferative disorders can be summarised as follows:

- (1) Markers are essential to distinguish:
 - (A) immature/acute lymphoblastic leukaemias (ALL), which, with the exception of Burkitt's or L3-ALL, are as a rule terminal deoxynucleotidyl transferase (TdT) positive, from mature/chronic lymphoproliferative disorders which are TdT negative. This is important when the clinical and cytological features of a chronic lymphoproliferative disorder resemble those of acute leukaemia, as may be the case of large cell lymphomas in leukaemic phase.³
 - (B) B cell from T cell lymphoid neoplasms.

(2) Immunophenotyping permits the establishment of clonality in B cell malignancies and the distinction from non-neoplastic reactive lymphocytosis. This is achieved by demonstrating the expression of only one of the two types of the immunoglobulin (Ig) light chain (κ or λ) on the surface or in the cytoplasm of the lymphocytes. By contrast, in

reactive conditions, both κ and λ positive B cells will be present.

There is no immunological marker to demonstrate clonality in T cells; however, aberrant phenotypes which are not present or are very uncommon among normal T cells, are suggestive of a clonal T cell proliferation.⁴

(3) Markers are useful to confirm or establish the diagnosis of a particular disease entity. Despite the lack of specificity of markers for the different lymphoid disorders, there are some well defined phenotypes such as that of B cell chronic lymphocytic leukaemia (B-CLL) and hairy cell leukaemia⁵ which are of great value for the recognition of these diseases, particularly when other clinical and laboratory features are atypical.

Recommendations

(1) SPECIMENS:

Peripheral blood specimens are often more suitable than bone marrow for immunophenotyping chronic lymphoproliferative disorders. Immunophenotyping of bone marrow cells is indicated when only the bone marrow is infiltrated and there are few or no abnormal cells in the peripheral blood.

(2) IMMUNOPHENOTYPING TECHNIQUES:

Immunophenotyping in mature lymphoproliferative disorders can be carried out either by immunofluorescence on unfixed cells in suspension or by immunoenzymatic methods on fixed cells spread on slides. The use of techniques applied to cell suspensions is recommended as the routine methodology for the following reasons:

(A) Information is provided on the density of expression of an antigen on the cells. This is very important when investigating the expression of immunoglobulin or of antigens recognised by certain monoclonal antibodies, such as CD22, which discriminate phenotypes typical of B-CLL from those seen in other B cell disorders.

(B) Some antigens such as that recognised by the monoclonal antibody FMC7 are usually destroyed by fixatives and therefore findings will be reliable only when they relate to unfixed cells in suspension. On the other hand, immunoenzymatic techniques may be helpful and are recommended in two situations:

- (i) in cases of plasma cell leukaemia which, as a rule, are negative with most immunological

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markers when tested in suspension, whereas the testing of fixed cells permits the demonstration of cytoplasmic immunoglobulin; (ii) when B cell clonality is not shown by membrane staining with anti- κ or anti- λ reagents as a consequence of the strong membrane expression of Fc γ receptors, which non-specifically bind the anti-immunoglobulin reagents. These are usually blocked by the fixative used in the immunoenzymatic methods.

Methodology for immunophenotyping

Recommendations for immunofluorescence and immunoenzymatic techniques in the study of chronic lymphoproliferative disorders are described below and are identical to those given in the study of acute leukaemias except for the detection of surface immunoglobulins (SmIg) heavy and light chains.

Peripheral blood or bone marrow are collected in anticoagulant (such as heparin or EDTA) and mononuclear cells are obtained by Ficoll density centrifugation. The cells are washed twice in phosphate buffered saline (PBS) and resuspended in PBS containing 1% bovine serum albumin (BSA) (Sigma), 0.05% sodium azide, and 2% human AB serum (pH: 7.6) (PBS-BSA-azide-AB buffer) except for the staining of SmIg.

(1) IMMUNOFLUORESCENCE ON CELL SUSPENSIONS

For the detection of cell membrane antigens by indirect immunofluorescence, 50 μ l of a cell suspension (10^6 cells) are incubated with an appropriate volume (ranging from 5 to 50 μ l) of optimally diluted monoclonal antibody for 30 minutes at 4°C. After two washes in PBS-BSA-azide-AB buffer the cells are incubated with the fluorescein-conjugated (FITC) anti-mouse Ig F(ab)₂ fragment (optimally diluted) for 30 minutes at 4°C, and washed twice in the buffer. The cell pellet is resuspended with two drops of PBS/glycerol (1 in 1), mounted on a glass slide, covered with a coverslip and sealed to be evaluated by a fluorescence microscope. An anti-fade reagent, such as 1,4-diazabicyclo-(2.2.2)-octane, may be added to the mounting medium to facilitate the reading under the microscope. A phase contrast microscope with adequate filters is recommended and at least 200 cells should be analysed for the fluorescence stain. A reaction is considered positive when the cell has multiple fluorescent dots on the membrane.

The same procedure is applied for flow cytometry but the cell pellet instead of being resuspended in glycerol/PBS, is resuspended in 500 μ l of Isoton and assessed on the flow cytometer. If analysis of the sample is performed 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 also be tested by flow cytometry, except for the detection of SmIg. The manufacturer's instructions for the lysing

procedure should be followed. Usually, 100–200 μ l of blood are incubated with the monoclonal antibody and subsequently with the FITC antimouse immunoglobulin. The methodology is similar to that described above but the buffer does not contain human AB serum and the pellet is treated with 1 ml lysing solution for 10 minutes at room temperature following the immunostaining. This is followed by two washes in PBS-BSA-azide buffer. Reading is performed in the flow cytometer after resuspending the pellet in 250 μ l of Isoton.

As in biphenotypic acute leukaemias, it may be useful in some circumstances to apply a double immunolabelling technique to determine if a single cell population coexpresses two different antigens in the membrane. For example, it may be informative to investigate the coexpression of a B cell marker (CD19) and CD5 in cases of B-CLL or that of CD4 and CD8 in T cell proliferations. Usually, this is performed by a direct immunofluorescence technique using two monoclonal antibodies directly conjugated to two fluorochromes (such as fluorescein and phycoerythrin). The monoclonal antibody should be independently titrated and the methodology is similar to that of indirect immunofluorescence with omission of the second incubation step.

In general terms, the use of the indirect immunofluorescence as the routine method is recommended for the following reasons:

- (A) the direct technique is less sensitive for the detection of an antigen when the expression in the membrane is weak;
 - (B) some monoclonal antibodies are not commercially available as reagents directly conjugated to fluorochromes; and,
 - (C) the indirect method is less expensive.
- The direct immunofluorescence method is recommended when double immunolabelling is performed.

Negative control preparations are always set up by replacing the monoclonal antibody with a mouse immunoglobulin of the same isotype and, when possible, positive controls are carried out on normal or leukaemic samples. For practical purposes, a mixture of mouse immunoglobulin of the various isotypes can be used as a negative control.

(2) SURFACE IMMUNOGLOBULIN (SMIG) STAINING

The methodology for the detection of immunoglobulin (heavy and light chains) in the cell membrane is similar to that described above, with slight modifications. Before the immunological reaction, the isolated cells are resuspended in tissue culture medium and incubated at 37°C for 30 to 60 minutes to remove extrinsic immunoglobulin bound by the Fc γ receptors present on B cells. Following this, the cells are washed once in PBS with 0.2% BSA and are subsequently stained with the anti-immunoglobulin reagents following standard techniques except that the buffer used for all washes does not contain 2% human AB serum.

**(3) IMMUNOALKALINE PHOSPHATASE
ANTIALKALINE PHOSPHATASE (APAAP) ON
FIXED CELLS⁶**

This may be carried out on smears or on a layer of mononuclear cells on a cyt centrifuge slide for all monoclonal antibodies except for the detection of immunoglobulin expression which should always be carried out on isolated mononuclear cells. The location of the cells is marked by encircling the area with a glass pencil. Smears and cyt centrifuge slides should be air-dried for at least six hours and up to 48 hours before the test. Fixation is done in **pure cold 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 µl of optimally diluted monoclonal antibody for 30 minutes. From now on the cells must not be allowed to dry and all the steps are carried out at room temperature. After a wash with 0·05 molar TRIS-buffered saline (TBS) (pH 7·6), the slides are incubated with 50 µl of a rabbit anti-mouse immunoglobulin diluted 1 in 20 in TBS and 2% human serum for 30 minutes, washed again in TBS, and incubated in the immunoalkaline phosphatase anti-alkaline phosphatase (APAAP) complexes diluted 1 in 60 in TBS for 45 minutes. Following a wash in TBS the 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, still wet, are mounted with Glycergel (Dako) and assessed under an ordinary microscope. Cells positive with a monoclonal antibody will be seen with red stain in the cytoplasm or on the cell surface.

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

Interpretation of results

Results have to be considered in the context of the cell morphology, and the proportion of leukaemic cells has to be estimated in the samples analysed. Therefore, the cutoff point to consider a marker positive will vary. For instance, for samples from primary leukaemias such as B-CLL or prolymphocytic leukaemia (PLL), which usually contain over 80% leukaemic cells, a cutoff point of 30% of cells stained with a marker can be considered to be positive. By contrast, in diseases such as non-Hodgkin's lymphomas in leukaemic phase or hairy cell leukaemia, the proportion of malignant cells ranges from a minority to large numbers. Therefore, results must be interpreted in each case and for each particular marker, according to whether the marker is or is not expressed in normal blood lymphocytes. For instance, 10% of cells positive with B-ly-7, which is consistently negative in normal lymphocytes, can be considered a positive finding; the interpretation will be different with the marker CD2 which is positive in most normal blood T lymphocytes.

The lack of specificity of most of the markers also needs to be considered and results must be interpreted taking into consideration findings with the whole panel of markers investigated and not of a marker in isolation. For example, CD5 is a T cell marker but is also positive in B-CLL cells and in a proportion of B cell non-Hodgkin's lymphoma; CD38 is positive in activated T cells but also in lymphoplasmacytic cells and plasma cells.

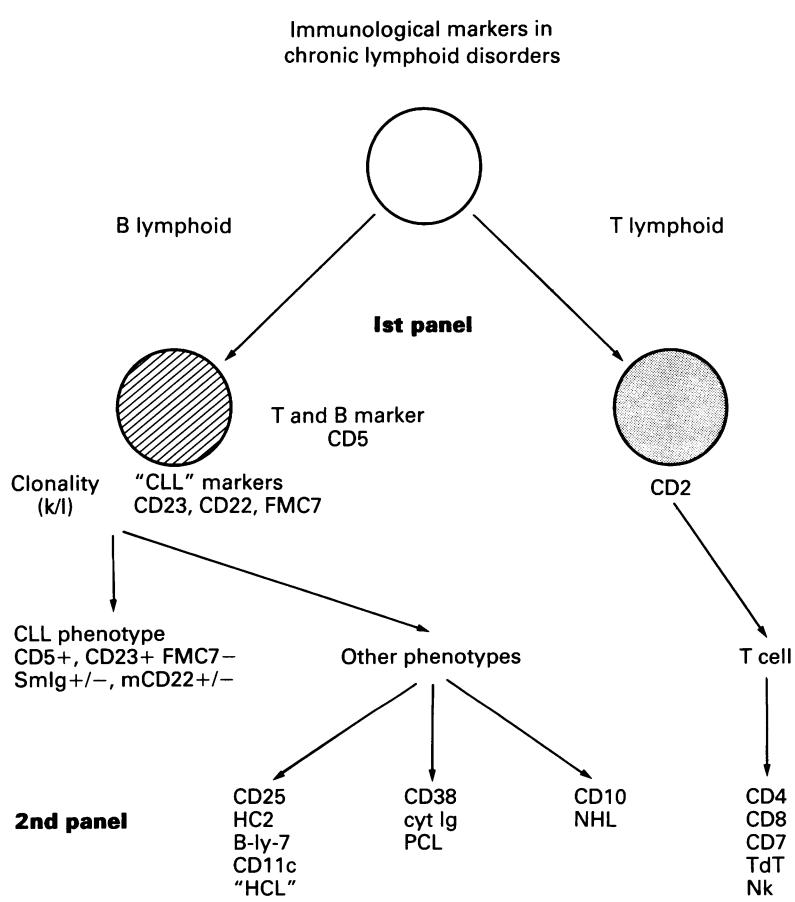


Diagram illustrating the panel of markers useful for the diagnosis of chronic lymphoproliferative disorders.

Recommended panel of monoclonal antibodies for the characterisation of chronic lymphoproliferative disorders

The first panel of markers (figure) to be tested is aimed at: distinguishing B from T cell disorders; assessing clonality in B cell proliferations; distinguishing a phenotype typical of B-CLL from those seen in other B lymphoproliferative disorders.

FIRST PANEL OF MARKERS

This **first panel** should include the following reagents:

A pan-T marker: CD2.

A pan-B marker: CD20 or CD19 or CD37 (the latter monoclonal antibody also weakly positive in a subset of T cells).

A marker expressed on T cells and a subset of

B cells (most B-CLL and few lymphomas): CD5.

Two markers to assess clonality: anti-immunoglobulin light chains: anti- κ and anti- λ .

Three markers which will help to discriminate between B-CLL and other B cell conditions: CD23, FMC7, and CD22.

With the use of these eight reagents in the first screen it is possible to distinguish:

T cell disorders: CD2+, CD5+/-, pan-B-, anti- κ/λ - from B cell disorders: CD2-, pan-B+, anti- κ or anti- λ +, CD5+/-.

A typical B-CLL phenotype (CD2-, CD5+, anti- κ or anti- λ weakly positive, pan-B+, CD23+, FMC7-, mCD22- or weakly positive) from phenotypes seen in other B cell malignancies (CD2-, CD5+/-, anti- κ or anti- λ strongly positive, pan-B+, CD23-, FMC7+, mCD22+).^{7,8}

SECOND PANEL OF REAGENTS

The cell reactivity with a second panel of reagents (figure) should be investigated when the first panel indicates a phenotype consistent with a T cell disorder or with a clonal B cell disorder with a marker profile atypical for B-CLL. In contrast, if the first panel shows an immunological profile characteristic of B-CLL, no further immunophenotyping is needed.

In the cases with a **T cell phenotype** (CD2+, CD5+/-), the use of the following monoclonal antibody is recommended^{2,4}:

CD3, CD7, CD4 and CD8 to confirm the T cell nature of the lymphocytes, see whether a particular subset (for example, CD4 or CD8) is expanded and whether leukaemic cells have a phenotype atypical among normal T lymphocytes.

TdT and CD1a to distinguish between thymic or immature (TdT+, CD1a+/-) lymphoid neoplasms (such as T cell acute lymphoblastic leukaemia) from post-thymic or mature (TdT-, CD1a-) T cell proliferations.

Optional markers which are sometimes of relevance are: (A) a monoclonal antibody which recognises antigens of natural killer and killer cells (CD11b, CD16, CD56 and CD57) and which may provide some information in the study of proliferations of large granular lymphocytes and, (B) those which recognise antigens in activated T cells (CD25, CD38 and class II HLA-DR determinants) in the analysis of suspected adult T cell leukaemia lymphoma and large cell T lineage non-Hodgkin's lymphomas.

In the cases in which the first panel shows a **clonal B cell disorder** with a phenotype **atypical for B-CLL**, the second panel of markers to be used needs to be tailored according to the diagnosis suspected from clinical features and cell morphology. For instance, if a diagnosis of hairy cell leukaemia is entertained, the monoclonal antibody panel B-ly-7, CD25, CD11c and HC2 will be useful to confirm the diagnosis and distinguish hairy cell leukaemia from other B cell conditions, such as the hairy cell leukaemia variant,^{5,9,10} or splenic lymphoma with villous lymphocytes¹¹ with which it may be confused.

Optional markers which sometimes give relevant information are CD10 when a diagnosis of follicular lymphoma in leukaemic phase is suspected¹² and CD38 in cases in which lymphoplasmacytic differentiation or plasma cell leukaemia is suspected from morphological examination.

A situation which may arise is that in which all the markers tested in the first panel are negative. The diagnosis of plasma cell leukaemia should then be suspected and tests on fixed cells to detect cytoplasmic immunoglobulin light chains and tests with CD38 should be carried out.¹³

The most characteristic immunophenotypic profiles on the various chronic B and T lymphoproliferative disorders are shown in tables 1 and 2.

Table 1 Immunological markers in mature B cell disorders

Marker	Leukaemias					Lymphomas		
	CLL	B-PLL	HCL	HCL-V	PCL*	SLVL	FL	McL
Smlg	Weak	Strong	Strong	Strong	-	Strong	Strong	Strong
CD5	++	-/+	-	-	-	-/+	-/+	++
Pan-B	++	++	++	++	-	++	++	++
CD23	++	-	-	-	-	-/+	-/+	-/+
FMC7	-/+	++	++	++	-	++	++	+
mCD22	Weak or -	++	++	++	-	++	++	+
CD25†/HC2	-	-	++	-	-	-/+	-	-
B-ly-7	-	-	++	+	-	-/+	-	-

*Expression of cytoplasmic immunoglobulin (light chain restricted) and CD38.

†CD25 may be identified in other conditions when using sensitive techniques.

CLL = chronic lymphocytic leukaemia; B-PLL = B cell prolymphocytic leukaemia; HCL = hairy cell leukaemia; HCL-V = hairy cell leukaemia variant; PCL = plasma cell leukaemia; SLVL = splenic lymphoma with villous lymphocytes; FL = follicular lymphoma; McL = mantle-cell lymphoma.

- = negative or positive in less than 10% of cases; -/+ = positive in 10 to 25% of cases;

+= positive in 25 to 75% of cases; ++ = positive in more than 75% of cases.

Table 2 Immunological markers in mature T cell disorders

Marker	T-PLL	ATLL	SS	LGL-L	T-NHL
CD2/CD5	++	++	++	++	+
mCD3*	+	+	++	++	+
CD7	++	-/+	+	+	+
CD4	++	++	++	-/+†	+
CD8	+**	-	-	++	+**
CD25	-/+	++	-/+	-	+

All cases have a mature (TdT-, CD1a-) phenotype.

*mCD3 = membrane expression. All cases express CD3 in the cytoplasm.

†Coexpressed with natural killer markers.

**Coexpressed with CD4 or with negative CD4.

T-PLL = T cell prolymphocytic leukaemia; ATLL = adult T cell leukaemia lymphoma; SS = Sézary's syndrome; LGL-L = large granular lymphocyte leukaemia; T-NHL = post-thymic T cell non-Hodgkin's lymphoma.

- = negative or positive in less than 10% of cases; -/+ = positive in 10 to 25% of cases;

+= positive in 25 to 75% of cases; ++ = positive in more than 75% of cases.

Indications for immunophenotyping

Immunophenotypic analysis is indicated on blood or bone marrow samples from all chronic lymphoproliferative disorders, including:

- (A) primary lymphoid leukaemias;
- (B) leukaemia or lymphoma syndromes, or non-Hodgkin's lymphoma in leukaemic phase when there are at least 10% circulating abnormal cells;
- (C) non-Hodgkin's lymphoma with bone marrow infiltration.

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