

Revised guideline on immunophenotyping in acute leukaemias and chronic lymphoproliferative disorders

**B. J. BAIN,
D. BARNETT,
D. LINCH,
E. MATUTES,
J. T. REILLY**

Guideline issued by the General Haematology Task Force of the British Committee for Standards in Haematology (BCSH), British Society of Haematology, 2 Carlton House Terrace, London

In 1994, the General Haematology Task Force (GHTF) of the British Committee for Standards in Haematology (BCSH) published two guidelines on immunophenotyping in acute leukaemias (General Haematology Task Force of the BCSH, 1994a) and chronic lymphoproliferative disorders (General Haematology Task Force of the BCSH, 1994b).

Over the past five years there have been major technical advances in this field (Table 1). In addition, some new monoclonal antibodies (McAb) have been shown to be highly specific for the lymphoid or myeloid lineages, respectively, and their use is therefore recommended in the diagnosis of acute leukaemias.

This revised guideline will focus on new techniques and reagents and define new panels of McAb recommended for lineage assignment in acute leukaemias and for the characterization of chronic lymphoproliferative disorders. This guideline does not consider in any detail the detection of minimal residual disease, the diagnosis of biphenotypic acute leukaemias or the evaluation of stem cell harvest. Nor does it address the selection of antibodies for therapeutic purposes. Furthermore, the antibody panels outlined here are not absolute and other McAb may be equally appropriate.

This guideline was written by a working party comprised of five members selected by the BCSH because of their expertise in this field. In addition the draft guideline was reviewed by eight UK laboratories in the forefront of the field of immunophenotyping. Their comments were discussed and incorporated into the draft when appropriate. The final manuscript was approved by all members of the BCSH and by the Committee of the

British Society of Haematology (BSH). The guideline represents the opinion of the BCSH Task Force and has been approved by the BSH.

Sample collection and conditions of storage

Peripheral blood and/or bone marrow samples should be collected and placed in an anticoagulant, either 0.34 M di- or tri-potassium ethylenediamine tetra-acetic acid (K₂ or K₃ EDTA) or preservative-free heparin. The choice of one or other anticoagulant will depend on the additional tests to be performed on the same specimen. For instance, heparin will be the anticoagulant of choice if cytogenetic analysis is to be performed while EDTA gives better preservation of the cell morphology. There is no need for anticoagulation of specimens such as pleural, ascitic or cerebrospinal fluid. For samples referred to central laboratories, fresh blood or marrow smears should be made at the time of collection.

If the sample is not tested in the local laboratory but referred to a central laboratory it should arrive within 24 h of collection and should be kept at room temperature (between 10 and 30 °C) during transportation. Suspected cases of Burkitt's lymphoma/leukaemia and cerebrospinal fluid samples require rapid transport and processing of the sample. It is recommended that a viability test, e.g. trypan blue exclusion test (Sigma Chemical Co., St. Louis, USA), be performed on samples received for immunophenotyping beyond 24 h (e.g. 24–72 h) to ensure that there is a good viability, e.g. at least 80% viable cells (Braylan *et al.*, 1997). In this situation results should be assessed with caution as antigen loss, particularly of markers which are weakly expressed, may occur. Addition of tissue culture medium is recommended if the specimens are likely to be stored beyond 24 h.

Table 1. Technical advances in immunophenotyping by flow cytometry

1. Application of double and triple immunostaining using monoclonal antibodies directly conjugated to fluorochromes.
2. Development of techniques permitting the use of whole blood and bone marrow rather than isolated mononuclear cells.
3. Development of reagents and techniques for permeabilizing cells to permit detection of nuclear and cytoplasmic antigens by flow cytometry.
4. Development of gating strategies with the use of CD45 to permit study of selected cell populations.
5. Development of techniques for quantification of surface and intracellular antigens.

General Recommendations

- Results need to be interpreted in the context of the clinical features and cell morphology, particularly in specimens containing a mixture of normal and neoplastic cells. When reporting on the marker results, the whole composite phenotype should be taken into account as most immunological markers are not strictly lineage specific; for instance cluster designation (CD)7 is a T-cell marker but it is also expressed in a proportion of cases of acute myeloid leukaemia (AML).
- Controls. Positive and negative controls for each McAb should be carried out only if a small number of tests are performed infrequently or if a specific antigen is used infrequently, to monitor the validity of the immunostaining and the lysing procedures. This is essential when a new McAb and/or batch of reagents is introduced, after an instrument service or calibration and/or when a new technique is applied. Positive and negative controls should be either leukaemic or normal cells by which the antigen is known to be expressed or not expressed. Normal residual cells within the sample may also act as an internal control. Laboratories with high workloads and well characterized McAb will acquire evidence of positive and negative controls in the range of clinical samples tested.
- Directly conjugated McAb are recommended for flow cytometry when available. Optimal dilutions of McAb and second layer reagents should be defined in each laboratory. Titration should be carried out using known positive and negative controls. Whenever possible, the manufacturer's guidance on the use of McAb should be followed. If dilution is undertaken, relevant documentation of experimental evidence supporting the use of antibodies at the relevant dilution should be available.
- Before setting up the techniques for immunophenotyping, the laboratory worker should spend a period of

training in a department with experience of immunophenotyping. Competency should be documented.

- There is no consensus on the cut-off point for considering a sample to be positive with a marker but commonly used criteria are: positivity in greater than 20% of leukaemic cells in acute leukaemias and positivity greater than 30% of leukaemic cells in chronic lymphoproliferative disorders. Although these cut-off points are arbitrary, they have been used by many groups. In certain circumstances, expression of a marker on the neoplastic or leukaemic cells is of interest rather than the expression on the total mononuclear cell fraction. This is the case with regard to the expression of CD5 in B cells from chronic lymphoid disorders and the expression of kappa and lambda on a minor B-cell population.
- It is essential that laboratories participate in an external quality assurance (EQA) program.

New techniques and applications

Use of whole blood or bone marrow

Flow cytometry can be applied to blood or bone marrow samples without the need to isolate mononuclear cells, thus simplifying laboratory procedures and making immunophenotyping of high risk, e.g. HIV-positive, samples safer. The samples should be treated with a hypotonic erythrocyte lysing solution with NH₄Cl-based reagents which causes minimal selective loss of cell populations. Care has to be taken in the lysing procedure as prolonged exposure to lysing agents may cause changes in the forward and side light scatter (SSC) patterns resulting in selective loss of certain populations, whilst inadequate exposure to lysing agents leaves some intact red cells, an excess of debris and makes results inaccurate.

Detection of intracellular (cytoplasmic and nuclear) antigens by flow cytometry

Cytoplasmic and nuclear antigens can be detected by flow cytometry using a variety of commercially available permeabilization/fixation solutions. Not all commercially available reagents are equally reliable for detecting intracellular and, in particular, cytoplasmic antigens. Careful attention is needed to ensure that these solutions do not affect the light scatter patterns, that they are suitable for routine use and that they can be combined to permit simultaneous detection of membrane and cytoplasmic/nuclear antigens. It should be noted that there are some solutions suitable for testing whole blood or bone

marrow specimens for nuclear antigens, e.g. terminal deoxynucleotidyl transferase (TdT), that are not reliable for cytoplasmic antigen detection.

A study evaluating four commercially available solutions: FACS Permeabilization solution (BD Biosciences, San Jose, CA, USA), Fix and Perm cell permeabilization kit (Ander Grub, Vienna), Optylise B-lysing solution (Immunotech, Marseille, France) and Permeafix (Ortho Diagnostic Systems, Raritan, NJ, USA) has shown that the four reagents have very minor effects on the light scatter pattern (Groeneveld *et al.* 1996) (Table 2). Fix and Perm and Permeafix gave similar results when detecting the nuclear enzyme TdT, cytoplasmic CD3 (cytCD3) and cytoplasmic immunoglobulin (cytIg) with results being comparable to those obtained by immunofluorescence microscopy (Pizzolo *et al.*, 1995; Groeneveld *et al.*, 1996). FACS Permeabilization solution and Optylise were reliable for the detection of TdT but gave equivocal results for cytCD3 and cytIg. Fix and Perm seems to be superior to Permeafix and other reagents for the detection of myeloperoxidase (MPO) by flow cytometry (Groeneveld *et al.*, 1996) (Table 2).

The technique for detection of intracellular antigens by single immunostaining involves:

- incubation of the specimens with the permeabilization/fixation solution for a variable period of time depending on the reagent (ranging from 10 min for Optylise B and FACS Permeabilization solution to 40 min for Permeafix),
- washing with a buffer composed of phosphate buffered saline (PBS) and bovine serum albumin (BSA) (PBS/BSA; pH: 7.3),
- incubation for 10–15 min with the fluorochrome-conjugated McAb against the antigen to be investigated, washing again in PBS/BSA and reading on the flow cytometer.

The time required to perform the test ranges from 40 min (FACS Permeabilization solution) to 80 min (Permeafix).

Table 2. Lysing solutions for the detection of intracytoplasmic and nuclear antigens*

Solution	Effect on light scatter	TdT	cytCD3	cytIg	MPO
Fix and Perm	None or minor	+	+	+	+
Permeafix	None or minor	+	+	+	–
FACS	None or minor	+	–	–	–
Permeabilization					
Optylise	None or minor	+	–	–	–

+, reliable; –, not reliable; * from Groeneveld *et al.* (1996).

For double immunostaining, e.g. detection of membrane and intracellular antigens, samples should first be processed using the standard technique to assess the expression of the surface marker. Ideally the membrane marker should be investigated using a fluorochrome-conjugated McAb involving only a single step incubation. Subsequently the samples are washed and processed further, as described earlier, to detect the intracellular marker. The use of phycoerythrin (PE)- instead of fluorescein (FITC)-conjugated McAb is recommended for the detection of antigens which are weakly expressed as PE gives a brighter signal and hence increased sensitivity.

Control preparations using isotype-matched fluorochrome-conjugated mouse immunoglobulins for membrane and cytoplasmic markers should be run in parallel. Control samples should be treated in the same way as the test sample. As for the standard immunophenotyping procedures, blocking of the Fc receptors should be performed when analysing isolated mononuclear cells but is not required when using whole blood or bone marrow specimens (General Haematology Task Force of the BCSH, 1994a).

The detection of intracellular antigens can be crucial for the diagnosis of acute leukaemias and rare cases of lymphoid disorders such as those arising from plasma cells. This is because the most specific markers for the myeloid and lymphoid lineages are only detectable in the cytoplasm and/or are present earlier in the cytoplasm than on the cell surface, e.g. CD3 for the T-lymphoid lineage (Janossy, Coustan-Smith & Campana, 1989), CD79a for the B-lymphoid lineage (Buccheri *et al.*, 1993) and antimyeloperoxidase (anti-MPO) for the myeloid lineage (Buccheri *et al.*, 1992).

In the past, intracellular staining could only be performed on fixed mononuclear cells spread on cytocentrifuge slides or on blood and bone marrow smears either using direct or indirect immunofluorescence and reading on a fluorescence microscope or applying an immunocytochemical technique (General Haematology Task Force of the BCSH, 1994a). Advances in sample preparation have permitted the estimation of the expression of intracellular and nuclear antigens by flow cytometry and thus have minimized subjective interpretation of the results and permitted a more accurate reading.

Gating strategies with McAb against CD45

This technique involves incubation of the samples with a fluorochrome-labelled CD45 McAb and with the McAb for which cell reactivity needs to be established labelled with an alternative fluorochrome. In the initial step of the

analysis, gating is set up on a CD45-positive vs. (SSC) dot plot. This procedure allows the identification of leucocytes and the exclusion of platelets and debris. Thereafter, another gating is performed which includes the cells positive with the McAb under study. (Borowitz *et al.*, 1993). A minimum number of CD45-positive events must be collected in order to obtain reliable results, particularly when estimating the numbers of low frequency cells such as CD34-positive cells (Barnett *et al.*, 1999). It should also be noted that there is a marked variation between laboratories as to the number of CD45-positive events collected – ranging from 10×10^3 to 10×10^6 . If gating with CD45 is required it is important to take into account the level of positivity of the antigen being tested in order to analyse a sufficient number of CD45-positive events. Gating on CD45-positive cells is of value for the estimation of CD34-positive stem cells in peripheral blood and bone marrow harvests used for allografting and autografting (Barnett *et al.*, 1999), for analysis of the abnormal plasma cell population in multiple myeloma or other plasma cell dyscrasias, for detection of rare populations and for the detection of failure of red cell lysis. However, CD45 gating is not usually required for routine diagnosis.

New antibodies (Table 3)

Over the last three or four years new McAb that are useful for the diagnosis of acute leukaemias or chronic lymphoid disorders have become available (Table 3). Their inclusion in either primary or secondary antibody panels is recommended for the routine diagnosis of acute leukaemias or

Table 3. Useful new monoclonal antibodies of established specificity

Cluster designation	Value
CD117	Better specificity for the myeloid lineage than CD13 and CD33 with reasonable sensitivity.
CD79a	High specificity and sensitivity for the B-lineage; it is expressed at all stages of B-cell differentiation including plasma cells.
CD79b	Specificity for B-lineage; reduced expression in chronic lymphocytic leukaemia and hairy cell leukaemia.
Anticyclin D1	Detection of increased levels of cyclin D1; mainly positive in mantle cell lymphoma and other cases with t(11;14)(q13;q32) but not completely specific.

chronic lymphoproliferative disorders. Among these antibodies are:

- CD79a McAb (e.g. mb-1), which detects an intracellular epitope of the alpha chain of the B-cell receptor. This is a functional protein in B lymphocytes which forms part of the B-cell antigen receptor and is highly specific for the B-lymphoid lineage. In addition to its specificity when tested by flow cytometry, CD79a has a high sensitivity as it is expressed from the earliest stages of B-cell differentiation all through the B-cell pathway up to the plasma cell stage, although clonal plasma cells from a proportion of cases of multiple myeloma are CD79a-negative. The majority of B-lineage acute lymphoblastic leukaemias (ALL) are CD79a-positive while reactions are usually negative in acute myeloid leukaemias (AML) (Buccheri *et al.*, 1993). Although there have been two reports describing CD79a expression in T lineage ALL (Pillozzi *et al.*, 1998; Lai *et al.*, 2000), the significance of these findings is still uncertain. One of the two studies (Lai *et al.*, 2000) documented three T-ALL cases whose cells expressed CD79a weakly. However, two of these cases were also CD10-positive qualifying as biphenotypic acute leukaemia (B plus T lymphoid) (Matutes *et al.*, 1997; Bene *et al.*, 1998b; Brunning *et al.*, 2001). The other report documented a 10% incidence of weak CD79a expression in a large series of cases of T-ALL using immunohistochemistry (Pillozzi *et al.*, 1998). Most of these positive cases were tested only with CD3 and CD79a; only a few were tested with CD1a. DNA analysis for the configuration of the immunoglobulin (Ig) heavy chain gene was not carried in these two studies and thus, the possibility that some of the cases correspond to leukaemias of early lymphoid progenitor cells not committed to the B or T lymphoid lineages could not be ruled out. The question of CD79a expression in T-ALL remains open though the above findings alert pathologists to potential diagnostic difficulties.
- CD117, a McAb that detects a 145 Kd tyrosine kinase transmembrane protein (c-kit), the receptor for stem-cell growth factor. The c-kit is expressed in a minority of haemopoietic cell precursors (< 4%), some of which are committed to the myeloid lineage, and in a minor proportion (< 1%) of thymocytes which are localized in the subcapsular cortex of the thymus and are able to differentiate *in vitro* into cells of myeloid or T-lymphoid lineage (de Castro *et al.*, 1994). An extensive study including close to 2000 samples from cases of acute leukaemia showed CD117 to be expressed in over two thirds of cases of AML regardless of the French–

American–British (FAB) subtype while less than 5% of cases of ALL were CD117-positive (Bene *et al.*, 1998a). Most of these latter cases either expressed other myeloid antigens, e.g. CD13 or CD33 and/or corresponded to early T (pro-T) ALL. The specificity of CD117 for the myeloid lineage is close to that of anti-MPO and far greater than that of CD13 or CD33 (Drexler, Thiel & Ludwig, 1991). The European Group for the Immunological Classification of Leukaemias (EGIL) has also stressed the value of CD117 in the recognition of apparently undifferentiated acute leukaemias which are likely to represent poorly differentiated acute myeloid leukaemias (Bene *et al.*, 1998a). CD117 is also an important myeloid marker for the definition of biphenotypic acute leukaemia. (Bene *et al.*, 1998b). It is recommended that CD117 be included in the panel of McAb for the routine immunophenotypic analysis of cases of acute leukaemias.

- CD79b, a McAb that detects an extracellular epitope of the B-cell receptor β chain and thus, is specific to B-cells. In the B-cell differentiation pathway, it is expressed later than CD79a, with a third of cases of B lineage ALL being CD79b-negative (Astsaturov *et al.*, 1996). These cases mainly correspond to pro-B and common-ALL. CD79b is of value in the diagnosis of chronic lymphoproliferative disorders because of its discriminatory power between chronic lymphocytic leukaemia (CLL), in which cells are negative or express membrane CD79b weakly when a PE-conjugated anti-CD79b McAb is used, and other B-cell diseases in which the cells usually show moderate or strong expression (Zomas *et al.*, 1996; Thompson *et al.*, 1997; Cabezudo *et al.*, 1999).
- McAbs that detect cyclin D1, which is preferentially, although not exclusively, expressed in cells from mantle cell lymphomas and other B-cell disorders which carry the t(11;14) translocation such as B-prolymphocytic leukaemia. However, cells from hairy cell leukaemia and from some B-cell lymphomas and CLL may express cyclin D1 weakly (de Boer C.J *et al.*,

1996). Expression of cyclin D1 can be detected by flow cytometry, as described by Elnenaï *et al.* (2001), with 85% specificity and sensitivity when compared with reverse transcriptase polymerase chain reaction (RT-PCR) for cyclin D1, D2 and D3 and with the presence of t(11;14) by fluorescence *in situ* hybridization (FISH). Nevertheless, problems in detecting cyclin D1 by immunohistochemistry or flow cytometry are not rare. Therefore, when mantle cell lymphoma is suspected and immunological results by either technique are negative, other techniques, e.g. for the detection of t(11;14) are required.

Panel of McAb for the diagnosis of acute leukaemias (Table 4 and Figure 1)

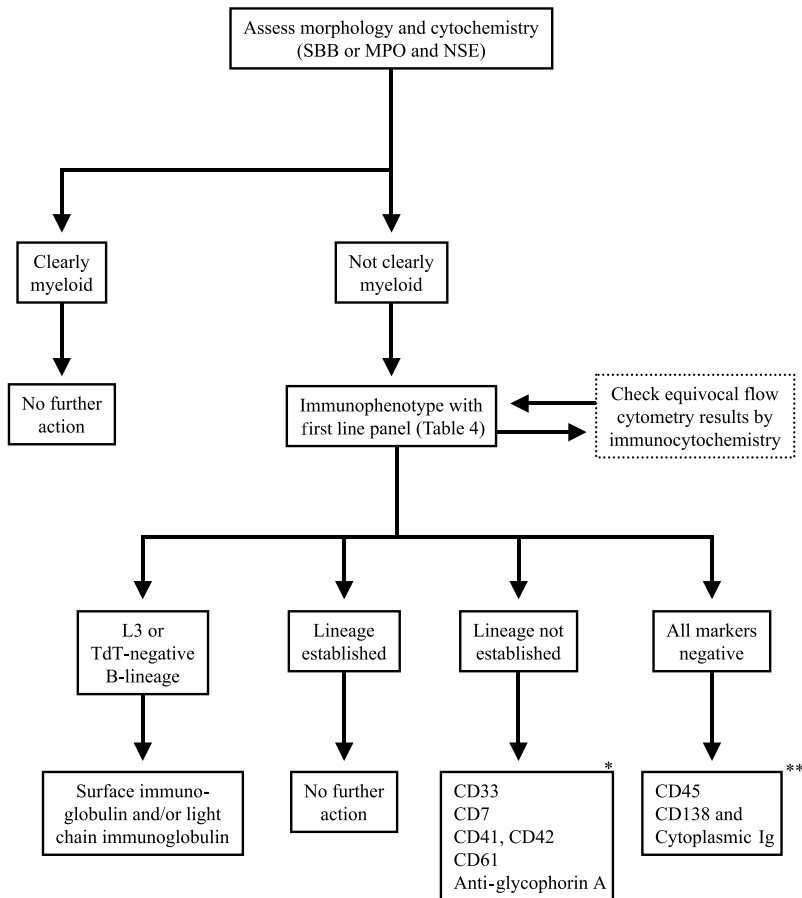
In the light of the new McAb and technologies available, the use of a revised panel for the characterization of cases of acute leukaemia is recommended (Table 4 and Figure 1).

The panel advised is largely based on the recommendations by the EGIL group (Bene *et al.*, 1995) with some modifications. Analysis comprises a two step process with the first panel of markers being applicable to all cases of acute leukaemia unless a clear myeloid commitment can be demonstrated by morphology and cytochemistry. If patients with AML are entered into a clinical trial, immunophenotyping is usually required for trial purposes. In other circumstances, immunophenotyping is indicated if it is not possible to establish a definitive diagnosis of AML on the basis of a Romanowsky stained film and standard cytochemistry. Immunophenotyping is essential in all cases of poorly differentiated myeloid leukaemia (M0-AML), megakaryoblastic leukaemias (M7-AML) and in some cases of monoblastic (M5a-AML) leukaemias and those with primitive erythroid cells as the predominant leukaemic cell. A second panel is selected, when necessary, to deal with specific diagnostic problems (Figure 1). Immunophenotyping may also be performed in cases of acute leukaemia with evidence of myeloid

Table 4 Panel of antibodies for the diagnosis of acute leukaemias

	B-lymphoid	T-lymphoid	Myeloid	Nonlineage restricted
First line:	CD79a*, CD22* CD19, CD10	CD3*, CD2	anti-MPO* CD117, CD13	TdT**
Second line:	SmIg (kappa/lambda) CytIg, CD138	CD7	CD33, CD41, CD42, CD61, glycophorin A	CD45, nonhaemopoietic markers

Optional markers: antilysozyme, CD14, CD15, CD36, HLA-Dr. *Cytoplasmic expression; **nuclear expression.



* In children, if only CD13 is positive, test for small cell tumours, e.g. neuroblastoma, rhabdomyosarcoma.

** In adults, test markers for nonhaemopoietic tumours, e.g. cytokeratin, and in children test for small cell tumours as above.

Figure 1. Immunophenotyping in acute leukaemias.

differentiation if required by trials and/or for research purposes.

Panel of antibodies: (McAb in bold indicate the changes made on the original guidelines recommended in 1994 by the GHTF of the BCSH):

First line

- Non-lineage restricted and/or stem cell marker: terminal deoxynucleotidyl transferase (TdT).
- Lineage-specific or lineage-associated markers:
B-lymphoid: **CD79a** cytoplasm (cyt), CD19, CD22 (cyt), CD10;
T-lymphoid: CD2, CD3 (cyt);
myeloid*: anti-MPO (cyt), **CD117**, CD13*. Among the myeloid associated McAb, CD13 was chosen instead of CD33 as the former detects a higher

proportion of AML cases (Legrand *et al.*, 2000; Baer *et al.*, 2001).

Second line

This panel will be used selectively, according to the results of the first line panel. The first line panel permits the diagnosis of the majority of cases of acute leukaemias and their classification into the three major subtypes: AML, B and T-lineage ALL and, the diagnosis of the majority of biphenotypic acute leukaemias (see appendix II for definition) whilst the second line panel is aimed at the identification of uncommon types of AML such as those with megakaryocytic or erythroid differentiation and at the exclusion or confirmation of a diagnosis of a non-haemopoietic malignancy.

The second line panel comprises:

- antibodies to Ig light chains if cells have a B-cell phenotype and TdT is negative;
- CD33, CD7, CD41, CD42, CD61 and anti glycoporphin A if the lineage is not established;
- CD45, cytIg, CD138 and markers for nonhaemopoietic tumours if results with the first and second line panels are negative and, in children, if the neoplastic cells express only CD13.

Optional McAb

- Antilysozyme: this is a McAb that detects lysozyme in the cytoplasm and although is not specific for monoclastic leukaemias, it is preferentially expressed in this subtype and may be more sensitive than CD14.
- CD14 against granulocytic and monocytic cells.
- CD15, which is characteristically positive in the subset of pro-B-ALL (CD79a-positive, CD19-positive, CD22-positive, CD10-negative, cytoplasmic IgM-negative) with 11q23 rearrangement and therefore its expression in ALL may be indicative of such a chromosomal abnormality.
- CD36 which is positive in early erythroid acute leukaemias. Although it is not specific for this lineage, as it may be positive in monoblasts, when the remaining immunophenotypic profile of the blast cells is considered (e.g., negative HLA-Dr and granulocytic markers) its expression supports a diagnosis of erythroid leukaemia.
- HLA-Dr which is expressed in: B-lineage ALL, a minority of cases of T-lineage ALL and most AML, but is characteristically negative in promyelocytic and erythroid leukaemias and up to half of megakaryoblastic leukaemias.

Panel of McAb for the diagnosis of chronic lymphoproliferative disorders (Table 5; Figure 2)

This panel is essentially the same as the one previously recommended (General Haematology Task Force of the BCSH, 1994b) with minor modifications. As for the diagnosis of acute leukaemias, it comprises a first line panel of markers applied to all cases and a second line to be selectively applied if indicated by the results with the first line panel.

First line

- Pan-T cell marker: CD2 (CD2 is recommended instead of CD3 because a proportion of T-cell diseases, whether positive or not with natural killer-associated markers, are CD3 negative).
- B-cell lineage associated markers which include a pan-B marker (e.g. CD19), two B-cell restricted markers (CD23, FMC7), surface immunoglobulin expression with estimation of the fluorescence intensity and light chain restriction using antikappa and antilambda reagents and assessment of the fluorescence intensity of membrane CD22 or CD79b.
- T-cell and B-cell subset marker: CD5.

The first line panel will permit distinction of B-cell from T-cell neoplasms and, within the B-cell disorders, will demonstrate whether or not the immunophenotype typical of CLL is present (Table 6) (Matutes *et al.*, 1994a,b).

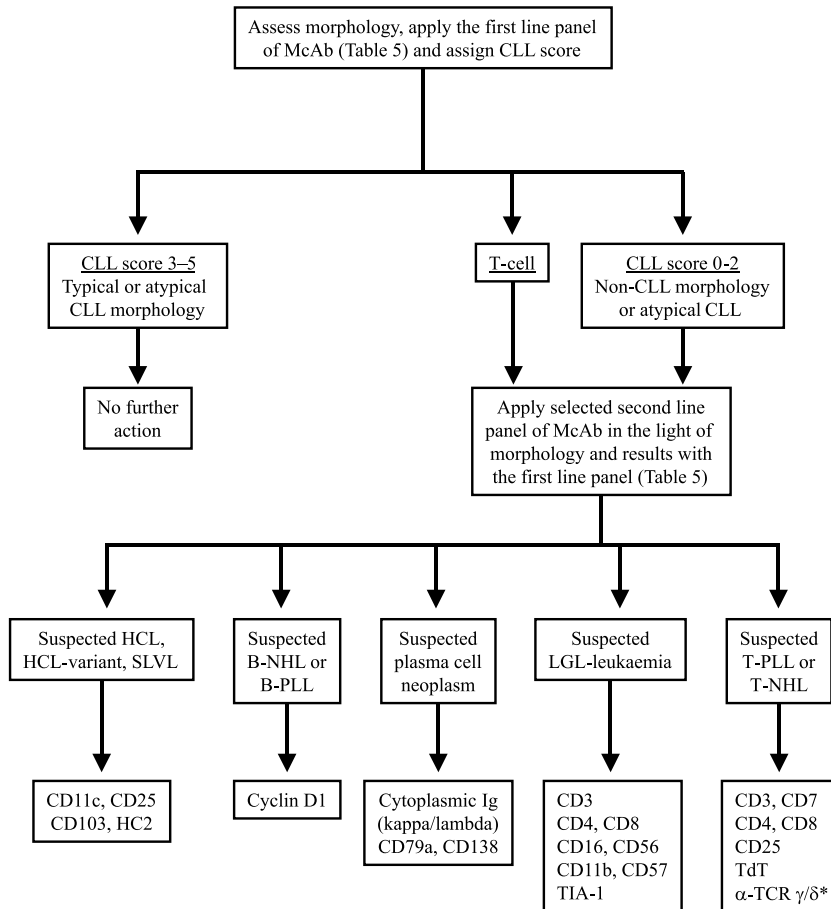
Second line panel

This will be applied selectively, depending on the cell morphology and the results with the first panel:

Table 5 Panel of markers for the diagnosis of chronic/mature lymphoproliferative disorders

First line:	B-cell CD19 CD23, FMC7, SmIg* (kappa/lambda) CD22*, CD79b*	T-cell CD2	B-cell and T-cell CD5	
Second line:	I CD11c, CD25 CD103, HC2	II CytIg (kappa/lambda) CD79a, CD138	III CD3, CD7 CD4, CD8 CD25	IV cyclin D1

*Intensity of membrane expression. I, disorders with villous cells; II, disorders with suspected lymphoplasmacytic or plasma cell differentiation; III, T-cell disorders; IV, suspected mantle cell and unclassifiable B-cell lymphomas. Optional markers: natural killer associated (CD16, CD56, CD11b, CD57); thymic markers (TdT); markers associated with activated T cells (CD25); cytotoxic T-cell or NK marker (TIA-1).



* If γ/δ hepatosplenic T-cell lymphoma is suspected

Figure 2. Immunophenotyping in chronic lymphoproliferative disorders. NHL, non-Hodgkin's lymphoma (other abbreviations are given in the text).

- CD11c, CD25, CD103 and HC2 in cases with circulating villous or hairy lymphoid cells and/or when a diagnosis of hairy cell leukaemia is suspected on clinical grounds. Although none of these markers are specific for hairy cell leukaemia, when assessed together they permit the distinction of typical hairy cell leukaemia (HCL) from cases of HCL-variant and splenic lymphoma with villous lymphocytes (SLVL) (Matutes *et al.*, 1994b).

- McAb anticyclin D1 if mantle cell lymphoma or B-cell prolymphocytic leukaemia (B-PLL) is suspected.
- Cytoplasmic expression of immunoglobulin heavy and light chains, cytoplasmic CD79a and membrane CD138 for cases in which neoplastic cells were negative with the first line panel of McAb and a lymphoplasmacytic and/or plasma cell proliferation is suspected on clinical and/or morphological grounds.
- CD3, CD4, CD7 and CD8 in cases in which the first line panel indicated a T-cell phenotype.

Table 6. Scoring system for the diagnosis of CLL

Marker	Score points	
	1	0
CD5	Positive	Negative
CD23	Positive	Negative
FMC7	Negative	Positive
SmIg	Weak	Strong
Membrane CD22/CD79b	Weak	Strong

Scores for CLL range from 3 to 5 and for non-CLL cases from 0 to 2. SmIg, surface immunoglobulin.

Optional markers

- Natural killer (NK) associated markers, e.g. CD16, CD56, CD57 and CD11b may be investigated in cases with a presumptive diagnosis of large granular lymphocyte (LGL) leukaemia, whether or not cells express T-cell specific markers such as CD3 or T-cell receptor (TCR). Use of these McAb is strongly recommended in those cases in which cells are CD2 positive but lack expression of specific T and B cell markers.

- TIA-1, a McAb that recognizes an intragranular protein in cytotoxic T lymphocytes and NK cells may be useful in cases in which an expansion of CD8-positive cells is documented as it may help to distinguish CD8-positive LGL-leukaemia from the minority of cases of other T-cell leukaemias that are CD8-positive, e.g. T-cell prolymphocytic leukaemia and Sezary syndrome which, unlike LGL leukaemia, are TIA-1 negative (Matutes *et al.*, 1996). In addition, this marker is characteristically expressed in a well defined subtype of T-cell lymphoma, the hepatosplenic γ/δ T-cell lymphoma, which arises from cells bearing the TCR γ/δ .
- Investigation of expression of the nuclear enzyme TdT is optional but is recommended in those cases shown to have a T-cell phenotype and immature or blastic morphology in order to exclude or confirm a diagnosis of T-lymphoblastic lymphoma/T-ALL.
- Markers associated with activated T-cells such as CD25 may be assessed in T-cell leukaemias and lymphomas in which the cells lack expression of most T-cell and NK associated markers and/or when a diagnosis of HTLV-I positive adult-T-cell leukaemia lymphoma is entertained. McAb anti-TCR γ/δ may be used if a diagnosis of hepatosplenic T-cell lymphoma is suspected.

The expression of the antigens detected by the McAb included in the panel, whether membrane or cytoplasmic, can be assessed by flow cytometry and direct or indirect immunofluorescence techniques following the guidelines outlined above. In cases in which sufficient material is not available for flow cytometry, an immunocytochemistry technique, such as the alkaline phosphatase antialkaline phosphatase (APAAP) method, can be carried out on bone marrow smears or cytopsin preparations, thus making optimal use of limited material.

The established and the potential roles of immunophenotyping in the diagnosis of acute leukaemias and chronic lymphoproliferative disorders is summarized in Table 7.

Appendix I

Quantitative flow cytometry

Several studies have highlighted the clinical and research applications of antigen quantification. Using such an approach, it has been possible to define antigen density on normal cells, monitor changes in antigen expression that occur during viral infection (Lenkei & Andersson, 1995) and ageing (Storie *et al.*, 1995) and also to facilitate leukaemia diagnosis and minimal residual disease moni-

Table 7. Current and possible future role of immunophenotyping in the diagnosis and management of haematological neoplasms

Established role of major clinical significance	Potential role
Diagnosis of ALL, M0 AML, M6 AML M7 AML and biphenotypic acute leukaemias.	Identification of poor prognosis subtypes of acute leukaemia, e.g. ALL with 11q23 rearrangement.
Demonstration of clonality in suspected B-cell lymphoproliferative disorders.	Detection of minimal residual disease in acute leukaemias and lymphoproliferative disorders.
Differential diagnosis of B and T lineage lymphoproliferative disorders and recognition of specific subtypes, e.g. hairy cell leukaemia.	Identification of M3 and M3 variant AML.
Quantification of stem-cells in peripheral blood and bone marrow harvests.	

oring (Lavabre-Bertrand *et al.*, 1994a; Lavabre-Bertrand *et al.*, 1994b; Farahat *et al.*, 1995a; Farahat *et al.*, 1995b; Caldwell, Patterson & Hakami, 1997). Several methods for antigen quantification have been developed: quantitative indirect immunofluorescence (QIFI) (Poncelet & Carayon, 1985), quantum simply cellular-antibody binding capacity (QSC-ABC) (Schwartz *et al.*, 1994), calibration of the flow cytometer with molecules of equivalent soluble fluorescence (MESF) and the subsequent use of antibody conjugates with known MESF/antibody ratios (Davis *et al.*, 1996; Iyer *et al.*, 1996), and, more recently, stabilized cellular immunofluorescence assay (SCIFA) (Janossy *et al.*, 1998).

Recent studies (Lavabre-Bertrand *et al.*, 1994b; Farahat *et al.*, 1995b) have employed quantitative flow cytometry to distinguish normal B-cell precursors from their leukaemic counterparts by identifying the differentially high expression of CD10 and CD19 in ALL. In addition, Ginaldi *et al.* (1996) have reported that the combined quantification of CD3 and CD7 expression is of diagnostic value in distinguishing normal from malignant T cells. Collectively, these studies suggest that such technology is of potential value in differentiating the neoplastic cell from its normal cell counterpart and may provide additional information with regard to the developmental process involved in lineage maturation.

However, despite the growing acceptance of antigen quantification techniques there are a number of important technical and quality control issues that have yet to be addressed. For example, two studies have reported discrepant findings for CD10 expression in B-lineage acute

lymphoblastic leukaemia (Lavabre-Bertrand *et al.*, 1994b; Farahat *et al.*, 1995b). In view of the increasing interest in antigen quantification, UK NEQAS for Leucocyte Immunophenotyping recently examined the technical issues involved with one particular technique, namely QSC-ABC. It was found that alterations in pH, incubation temperature, lysing reagent, fluorochrome and antibody titre can all have significant effects on the resulting antibody binding capacity (ABC) value (Barnett *et al.*, 1998). For example, the ABC for CD4 on normal lymphocytes was shown to be significantly lower at pHs above and below 7.4 (differences greater than 40 000 molecules/cell being noted) whilst for CD3, the ABC value obtained was dependent on the fluorochrome used and on whether single or multicolour analysis was undertaken. Furthermore, although when antigens are normally distributed (e.g. CD3 and CD4), no significant differences are observed in their ABC values when using either mean or median channel, significant differences are found for antigens that do not have a normal distribution such as CD8 (due to the presence of CD8^{dim} cells), when median channel values are consistently lower. Bikoue *et al.* (1996) have also shown that different monoclonal antibodies to a given antigen (e.g. CD8) can give different ABC values when the same cells are analysed.

Studies by Barnett *et al.* (1998) highlight the urgent need for a standard approach to enable intra- and interlaboratory comparisons. They concluded that, for the QSC-ABC method at least, single colour staining using FITC conjugated antibodies, with all reagents at pH 7.4 ± 0.1 and incubation and lysing at 20 ± 1 °C should be considered as the benchmark technique. Indeed, using this approach a high degree of interlaboratory concordance can be achieved for CD3, CD4, CD8 and CD19 ABC (Barnett *et al.*, 2000).

In conclusion, whilst antigen density determination appears to have some useful clinical applications, the techniques still require greater standardization before they should be introduced into the routine assessment of patients with leukaemia.

Appendix II

Potentially useful new antibodies

Potentially useful new antibodies that still need validation or for which a precise role has not yet been defined include the following (Table 8):

- the McAb 5E10 and PL-M3 that recognize the promyelocytic (PML) protein in cases of AML-M3 carrying

Table 8. Potentially useful monoclonal antibodies

Antibody	Potential value
5E10 or PL-M3	Recognition of altered distribution of PML protein in M3 and M3 variant AML.
7.1/NG2	Detection of ALL with 11q23 rearrangements and a proportion of CD56-positive AML with monocytic differentiation with or without abnormalities of 11q23.
Anti-TCR α/β Anti-TCR γ/δ	Sensitivity and high specificity for the T-cell lineage.
CD38	Poor prognostic marker in CLL.

the t(15;17)(q22;q11–12) and having rearrangement of PML-RARa. The pattern of PML distribution is specific and permits recognition of its altered distribution for these AML cases. (O'Connor *et al.*, 1997; O'Connor, Evans & Morgan, 1999). M3 AML shows either particulate nuclear or diffuse cytoplasmic staining in comparison with a smaller number of larger nuclear dots in normal promyelocytes or the myeloblasts of other AML subtypes. It has been suggested that PL-M3 is more specific than 5E10 (O'Connor, Evans & Morgan, 1999). Assessment of the pattern of distribution needs to be carried out by fluorescence microscopy or by immunocytochemistry (Falini *et al.*, 1997) as it is not possible by flow cytometry.

- The 7.1/NG2 McAb originally documented as being expressed in a subset of acute leukaemias, both lymphoblastic and myeloblastic, characterized by rearrangement of the 11q23 gene (Behm *et al.*, 1996; Smith *et al.*, 1996; Mauvieux *et al.*, 1999). As this subgroup of acute leukaemias carry a bad prognosis, it would be useful to be able to detect them by a simple technique such as flow cytometry. However, although a recent study confirmed that expression of this McAb in ALL is highly associated with pro-B-ALL with chromosomal abnormalities of 11q23, including t(4;11), t(9;11) and t(11;19), this McAb has been found to identify a subset of AML cases with monocytic differentiation and expression of CD56 but without evidence of 11q23 rearrangement (Wutcher *et al.*, 1998). Before routine use of this antibody can be recommended, a larger number of cases need to be investigated to determine its specificity.
- McAb that recognize the various chains of the TCR: anti-TCR α/β and anti-TCR γ/δ which are very specific for the T-lymphoid lineage. Although such antibodies

have been available for several years, they have not been widely applied in the diagnosis of acute leukaemias. German ALL trials have suggested that T-ALL cases with expression of TCR α/β fare much worse than those which express TCR γ/δ . In addition, expression of TCR provides evidence of T-lymphoid commitment in T-ALL and biphenotypic acute leukaemias.

- CD38, expressed in a variety of haemopoietic cells from different lineages. Although there is still disagreement as to whether CD38 is a surrogate marker for CLL with unmutated IgVH genes (Damle *et al.*, 1999; Damle *et al.*, 2000) or simply a marker of poorer prognosis CLL (Hamblin *et al.*, 1999; Hamblin *et al.*, 2000; Ibrahim *et al.*, 2001), an argument could be made for its inclusion in the panel of markers for chronic lymphoproliferative disorders.

Appendix III

Definition of biphenotypic acute leukaemia

Recognition of biphenotypic acute leukaemias is relevant because of the poor outcome of these patients and its association with 'bad prognostic' cytogenetic markers (Carbonell *et al.*, 1996; Legrand *et al.*, 1998; Killick *et al.*, 1999). Definition of biphenotypic acute leukaemia by using objective criteria is important in order to distinguish this unusual type of acute leukaemia (5% of cases) from ALL and AML with aberrant expression of a marker more characteristic of another lineage. The EGIL group has recommended a scoring system (Bene *et al.*, 1998a) with some modifications on the one previously proposed

Table 9. Scoring system for the diagnosis of biphenotypic acute leukaemias*

Score	B-lymphoid	T-lymphoid	Myeloid
2	CD79a cytCD22 cytIgM	CD3 anti-TCR α/β anti-TCR γ/δ	MPO**
1	CD19 CD20 CD10	CD2 CD5 CD8 CD10	CD117 CD13 CD33 CD65
0.5	TdT CD24	TdT CD7 CD1a	CD14 CD15 CD64

*Biphenotypic acute leukaemia is defined when scores for the myeloid and one of the lymphoid lineages are > 2 points.

** Demonstrated by the McAb anti-MPO or cytochemistry. Each marker scores the corresponding point.

(Matutes *et al.*, 1997). This is based on the number and its degree of lymphoid/myeloid specificity of the antigens expressed by the leukaemic cells (Table 9). Biphenotypic acute leukaemia is defined when scores for the myeloid and one of the lymphoid lineages are over two points; rare cases may show trilineage differentiation or a B plus T cell precursor phenotype (Matutes *et al.*, 1997). The criteria for the definition of biphenotypic acute leukaemia proposed here are those adopted in the WHO classification of haemopoietic malignancies (Brunning *et al.*, 2001).

References

- Atsaturov I.A., Matutes E., Morilla R., Seon B.K., Mason D.Y., Farahat N. & Catovsky D. (1996) Differential expression of B29 (CD79b) and mb-1 (CD79a): proteins in acute lymphoblastic leukaemia. *Leukemia* **10**, 769–773.
- Baer M.R., Steward C.C., Dodge R.K., Leget G., Sule N., Mrozek K., Schiffer C.A., Powell B.L., Kolitz J.E., Moore J.O., Stone R.M., Davey F.R., Carroll A.J., Larson R.A. & Bloomfield C.D. (2001) High frequency of immunophenotype changes in acute myeloid leukemia at relapse: implications for residual disease detection. *Blood* **97**, 3574–3580.
- Barnett D., Janossy G., Lubenko A., Matutes E., Newland A. & Reilly J.T. (1999) For the General Haematology Task Force of the British Committee for Standards in Haematology. Guideline for the flow cytometric enumeration of CD34+ haemopoietic stem cells. *Clinical and Laboratory Haematology* **21**, 301–308.
- Barnett D., Storie I., Granger V., Whitby L., Reilly J.T., Brough S., Garner S., Lawry J., Richards S., Bell A.E. & Shenton B.K. (2000) Standardisation of lymphocyte antibody binding capacity (ABC). A multicentre study. *Clinical and Laboratory Haematology* **22**, 89–96.
- Barnett D., Storie I., Wilson G.A., Granger V. & Reilly J.T. (1998) Determination of leucocyte antibody binding capacity (ABC): the need for standardization. *Clinical and Laboratory Haematology* **20**, 155–164.
- Behm F.G., Smith F.O., Raimondi S.C., Pui C.H. & Bernstein I.D. (1996) Human homologue of the rat chondroitin sulfate proteoglycan, NG2, detected by monoclonal antibody 7.1 identifies childhood acute lymphoblastic leukemias with t (4;11) (q21;q23) or t (11;19) (q23;p13) and MLL rearrangements. *Blood* **87**, 1134–1139.
- Bene M.C., Castoldi G., Knapp W., Ludwig W.D., Matutes E., Orfao A. & van't Veer M.B. for the European Group for the Immunological Characterization of Leukemias (EGIL) (1995) Proposals for the immunological classification of acute leukaemias. *Leukemia* **9**, 1783–1786.
- Bene M.C., Bernier M., Casasnovas R.O., Castoldi G., Knapp W., Lanza F., Ludwig W.D., Matutes E., Orfao A., Sperling C. & van't Veer M.B. for the European Group for the Immunological Classification of Leukemias (EGIL) (1998a). The reliability and specificity of c-kit for the diagnosis of acute myeloid leukemias and undifferentiated leukemias. *Blood* **92**, 596–599.
- Bene M.C., Castoldi G., Knapp W., Ludwig W.D., Matutes E., Orfao A. & van't Veer M.B. for the European Group for the

- Immunological Characterization of Leukemias (EGIL) (1998b) The value of c-kit in the diagnosis of biphenotypic acute leukaemia. *Leukemia* **12**, 203.
- Bikoue A., George F., Poncet L.W., Murin M., Janossy G. & Sampol J. (1996) Quantitative analysis of leukocyte membrane antigen expression: normal adult values. *Cytometry* **26**, 137–147.
- de Boer C.J., Kluin-Nelemans J.C., Dreef E., Kester M.G.D., Kluin P.M., Schuurin E. & van Krieken J.H.J.M. (1996) Involvement of the CCND1 gene in hairy cell leukemia. *Annals of Oncology* **7**, 251–256.
- Borowitz M.J., Guenter K.L., Shults K.E. & Stelzer G.T. (1993) Immunophenotyping of acute leukaemia by flow cytometric analysis. Use of CD45 and right-angle light scatter to gate on leukaemic blasts in three colour analysis. *American Journal of Clinical Pathology* **100**, 534–540.
- Braylan R.C., Borowitz M.J., Davis B.H., Stelzer G.T. & Steward C.C. (1997) US-Canadian consensus recommendations on the immunophenotypic analysis of haematologic neoplasia by flow cytometry. *Cytometry* **30**, 214–230.
- Brunning R.D., Matutes E., Borowitz M., Flandrin G., Head D., Vardiman J. & Bennett J. (2001) Acute leukaemias of ambiguous lineage. In: *WHO Tumours of Haematopoietic and Lymphoid Tissues* (eds E.S. Jaffe, N.L. Harris, H. Stein & J.W. Vardiman). IARC Press, Lyon, pp. 106–107.
- Buccheri V., Mihaljevic B., Matutes E., Dyer M.J.S., Mason D.Y. & Catovsky D. (1993) mb-1: a new marker for B-lineage lymphoblastic leukemia. *Blood* **82**, 853–857.
- Buccheri V., Shetty V., Yoshida N., Morilla R., Matutes E. & Catovsky D. (1992) The role of an anti-myeloperoxidase antibody in the diagnosis and classification of acute leukaemia. a comparison with light and electron microscopy cytochemistry. *British Journal of Haematology* **80**, 62–68.
- Cabezudo E., Morilla R., Carrara P. & Matutes E. (1999) Quantitative analysis of CD79b, CD5 and CD19 in B-cell lymphoproliferative disorders. *Haematologica* **84**, 413–418.
- Caldwell C.W., Patterson W.P. & Hakami N. (1997) Alterations of Hle-1 (T200) fluorescence intensity on acute lymphoblastic leukaemia cells may relate to therapeutic outcome. *Leukaemia Research* **11**, 103–106.
- Carbonell F., Swansbury J., Min T., Matutes E., Farahat N., Buccheri V., Morilla R., Secker-Walker L. & Catovsky D. (1996) Cytogenetic findings in acute biphenotypic leukemia. *Leukemia* **10**, 1283–1287.
- de Castro C.M., Denning S.M., Langdon S., Vandenberg G.R., Kurtzberg J., Scarce R., Haynes B.F. & Kaufmann R.E. (1994) The c-kit proto-oncogene receptor is expressed on a subset of human CD3-, CD4-, CD8-(triple negative) thymocytes. *Experimental Hematology* **22**, 1205–1233.
- Damle R., Wasil T., Allen S., Schulman P., Rai K.R. & Chlorazzi N. (2000) Updated data on Vgene mutation status and CD38 expression in B-cell. *Blood* **95**, 2456–2457.
- Damle R.N., Wasil T., Fais F., Ghiotto F., Valetto A., Allen S.L. & Buchbinder A. (1999) IgV status and CD38 expression as novel prognostic indicators in chronic lymphocytic leukaemia. *Blood* **94**, 1840–1847.
- Davis K.A., Abrams B., Hoffman R.A. & Bishop J.E. (1996) Quantitation and valence of antibodies bound to cells. *Cytometry* **8** (Suppl.), 125 (Abstract).
- Drexler H.G., Thiel E. & Ludwig W.D. (1991) Review of the incidence and clinical relevance of myeloid antigen-positive acute lymphoblastic leukemia. *Leukemia* **5**, 637–645.
- Elnenaei M.O., Jadayel D.M., Matutes E., Morilla R., Owusu-Ankomah K., Atkinson S., Titley I., Mandala E.M. & Catovsky D. (2001) Cyclin D1 by flow cytometry as a useful tool in the diagnosis of B-cell malignancies. *Leukemia Research* **25**, 115–123.
- Falini B., Flenghi L., Lo Coco F., Cordone I., Diverio D., Pasqualucci L., Biondi A., Riganeli D., Orleth A., Liso A., Mandelli M.F., Pellici P.-G. & Pileri S. (1997) Immunocytochemical diagnosis of acute promyelocytic leukemia (M3) with the monoclonal antibody PG-M3 (anti-PML). *Blood* **90**, 4046–4053.
- Farahat N., Lens D., Morilla R., Matutes E. & Catovsky D. (1995a) Differential TdT expression in acute leukaemia by flow cytometry: a quantitative study. *Leukemia* **9**, 583–587.
- Farahat N., Lens D., Zomas A., Morilla R., Matutes E. & Catovsky D. (1995b) Quantitative flow cytometry can distinguish between normal and B-cell precursors. *British Journal of Haematology* **9**, 640–646.
- General Haematology Task Force of the BCSH. (1994a) Immunophenotyping in the diagnosis of acute leukaemias. *Journal of Clinical Pathology* **47**, 777–781.
- General Haematology Task Force of the BCSH. (1994b) Immunophenotyping in the diagnosis of chronic lymphoproliferative disorders. *Journal of Clinical Pathology* **47**, 871–875.
- Ginaldi L., Matutes E., Farahat N., de Martinis M., Morilla R. & Catovsky D. (1996) Differential expression of CD3 and CD7 in T-cell malignancies: a quantitative study by flow cytometry. *British Journal of Haematology* **93**, 921–927.
- Groeneveld K., te Marvelde J.G., van den Beemd M.W.M., Hooijkaas H. & van Dongen J.J.M. (1996) Flow cytometric detection of intracellular antigens for immunophenotyping of normal and malignant leukocytes. BTS Technical Report. *Leukemia* **10**, 1383–1389.
- Hamblin T.J., Orchard J.A., Gardiner A. & Oscier D.G. (2000) Immunoglobulin V genes and CD38 expression in CLL. *Blood* **95**, 2455–2456.
- Hamblin T.J., Davis Z., Gardiner A., Oscier D.G. & Stevenson F.K. (1999) Unmutated Ig V (H) genes are associated with a more aggressive form of chronic lymphocytic leukemia. *Blood* **94** (6), 1848–1854.
- Ibrahim S., Keating M., Do K.-A., O'Brien S., Huh Y.O., Jilani L., Lenner S., Kantarijan H.M. & Albitar M. (2001) CD38 expression as an important prognostic factor in B-cell chronic lymphocytic leukemia. *Blood* **98**, 181–186.
- Iyer S., Suni M., Davis K. & Maino S. (1996) Quantitation of cellular expression of CD69 on activated T cells using R-phycoerythrin labelled beads. *Cytometry* **8** (Suppl.), 113 (Abstract).
- Janossy G., Bikoue A., Tilling R.S., Reilly J., Granger V. & Barnett D. (1998) The standardization of quantitative direct immunofluorescence: the stabilized cellular immunofluorescent analysis (SCIFA). *Cytometry* **9** (Suppl.), 145–146.
- Janossy G., Coustan-Smith E. & Campana D. (1989) The reliability of cytoplasmic CD3 and CD22 antigen expression in the immunodiagnosis of acute leukemia – a study of 500 cases. *Leukemia* **3**, 170–181.

- Killick S., Matutes E., Powles R.L., Treleaven J.G., Kulkarni S., Zomas A., Atra A., Swansbury J., Saso R., Hamblin M. & Catovsky D. (1999) Outcome of biphenotypic acute leukemia. *Haematologica* **84**, 699–706.
- Lai R., Juco J., Lee S.F., Nahirniak S. & Etches W.S. (2000) Flow cytometry detection of CD79a expression in T-cell acute lymphoblastic leukemias. *American Journal of Clinical Pathology* **113**, 823–830.
- Lavabre-Bertrand T., Duperray C., Brunet C., Poncelet P., Exbrayat C., Bourquard P., Lavabre-Bertrand C., Brochier J., Navarro M. & Janosy G. (1994a) Quantification of CD24 and CD45 antigens in parallel allows a precise determination of B-cell maturation stages. relevance for the study of B-cell neoplasms. *Leukemia* **8**, 402–408.
- Lavabre-Bertrand T., Janosy G., Ivory K., Peters R., Secker-Walker L. & Porwit-MacDonald A. (1994b) Leukemia-associated changes identified by quantitative flow cytometry. I. CD10 expression. *Cytometry* **18**, 209–217.
- Legrand O., Perrot J.-Y., Baudard M., Cordier A., Lautier R., Simonin G. & Zittoun R. (2000) The immunophenotype of 177 adults with acute myeloid leukemia: proposal of a prognostic score. *Blood* **96**, 870–877.
- Legrand O., Perrot J.-Y., Simonin G., Baudard M., Cadiou M., Blanc C., Ramond C., Viguie F., Marie J.P. & Zittoun R. (1998) Adult biphenotypic acute leukaemia: an entity with poor prognosis which is related to unfavourable cytogenetics and P-glycoprotein overexpression. *British Journal of Haematology* **100**, 147–155.
- Lenkei R. & Andersson B. (1995) High correlations of anti-CMV titers with lymphocyte activation and CD% & antibody-binding capacity as estimated with three-color, quantitative flow cytometry in blood donors. *Clinical Immunology and Immunopathology* **77**, 131–138.
- Matutes E., Coelho E., Aguado M.J., Morilla R., Crawford A., Owusu-Ankomah K. & Catovsky D. (1996) Expression of TIA-1 and TIA-2 in T-cell malignancies and T-cell lymphocytosis. *Journal of Clinical Pathology* **49**, 154–158.
- Matutes E., Morilla R., Farahat N., Carbonell F., Swansbury J., Dyer M. & Catovsky D. (1997) Definition of acute biphenotypic leukemia. *Haematologica* **82**, 64–66.
- Matutes E., Morilla R., Owusu-Ankomah K., Houlihan A., Meeus P. & Catovsky D. (1994a) The immunophenotype of hairy cell leukemia (HCL). Proposal for a scoring system to distinguish HCL from B-cell disorders with hairy or villous lymphocytes. *Leukemia and Lymphoma* **14** (Suppl.1), 57–61.
- Matutes E., Owusu-Ankomah K., Morilla R., Garcia Marco J., Houlihan A., Que T.H. & Catovsky D. (1994b) The immunological profile of B-cell disorders and proposal of a scoring system for the diagnosis of CLL. *Leukemia* **8**, 1640–1645.
- Mauvieux L., Delabesse E., Bourquelot P., Radford-Weiss I., Bannaceur A.-L., Flandrin G., Valensi F. & Macintyre E.A. (1999) NG2 expression in MLL rearranged acute myeloid leukaemia is restricted to monoblastic cases. *British Journal of Haematology* **107**, 674–676.
- O'Connor S.J.M., Evans P.A.S. & Morgan G.T. (1999) Diagnostic approaches to acute promyelocytic leukaemia. *Leukemia Lymphoma* **33**, 53–64.
- O'Connor S.J., Forsyth P.D., Dalal S., Evans P.A., Short M.A., Shiach C., Jack A.S. & Morgan G.H. (1997) The rapid diagnosis of acute promyelocytic leukemia using PML (5E10) monoclonal antibody. *British Journal of Haematology* **99**, 597–604.
- Pillozzi E., Pulford K., Jones M., Muller-Hermelink H.-K., Falini B., Ralfkiaer E., Pileri S., Pezzella F., Wolf-Peeters C., Arber D., Stein H., Mason D. & Gatter K. (1998) Coexpression of CD79a (JCB117) and CD3 by lymphoblastic lymphoma. *Journal of Pathology* **186**, 140–143.
- Pizzolo G., Vincenzi C., Nadali G., Veneri D., Vinante F., Chilosi M., Basso G., Connelly M.C. & Janosy G. (1995) Detection of membrane and intracellular antigens by flow cytometry following ORTHO PermeaFix fixation. *Leukemia* **9**, 226–228.
- Poncelet P. & Carayon P. (1985) Quantification of cell-surface antigens by indirect immunofluorescence using monoclonal antibodies. *Journal of Immunological Methods* **85**, 65–75.
- Schwartz A., Ottinger J., Wallace E., Poon R. & Fernandez-Repollet E. (1994) Quantitative determination of antibody binding capacity (ABC) by flow cytometry. *European Journal of Histochemistry* **38**, 13–20.
- Smith F.O., Rauch C., Williams D.E., March C.J., Arthur D., Hilden J., Lampkin B.C. & Buckley J.D. (1996) The human homologue of rat NG2, a chondroitin sulfate proteoglycan is not expressed on the cell surface of normal haemopoietic cells but is expressed by acute myeloid leukemia blasts from poor-prognosis patients with abnormalities of chromosome band 11q23. *Blood* **87**, 1123–1133.
- Storie I., Wilson G.A., Granger V., Barnett D. & Reilly J.T. (1995) Circulating CD20^{dim} T-lymphocytes increase with age: evidence for a memory cytotoxic phenotype. *Clinical and Laboratory Haematology* **17**, 323–328.
- Thompson A.A., Talley J.A., Do H.N., Kagan H.L., Kunkel L., Berenson J., Cooper M.D., Saxon A. & Wall R. (1997) Aberrations of the B-cell receptor B29 (CD79b) gene in chronic lymphocytic leukemia. *Blood* **90**, 1387–1394.
- Wutcher C., Schnittger S., Schoch C., Harbott J., Martin M., Sperling C., Karawajew L., Schonberger A., Dorken B. & Ludwig W.D. (1998) Detection of acute leukemia cells with 11q23 rearrangements by flow cytometry: sensitivity and specificity of monoclonal antibody 7.1. *Blood* **92** (Suppl. 1), 228a.
- Zomas A.P., Matutes E., Morilla R., Owusu-Ankomah K., Seon B.K. & Catovsky D. (1996) Expression of the immunoglobulin-associated protein B29 in B-cell disorders with the monoclonal antibody SN8 (CD79b). *Leukemia* **10**, 1966–1970.