Demonstration of cytoplasmic and nuclear antigens in acute leukaemia using flow cytometry

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Abstract

Aims—To detect cytoplasmic and nuclear antigens using flow cytometry in acute leukaemia and to use this technique for double marker combinations.

Methods—Cytoplasmic staining was carried out in samples from 40 cases of acute leukaemia with monoclonal antibodies against the myeloid antigen CD13, the lymphoid antigens CD3, CD22, μ chain and the enzymes terminal deoxynucleotidyl transferase (TdT) and myeloperoxidase (MPO). The cells were fixed with paraformaldehyde and permeabilised with Tween 20 and Becton Dickinson's FACS lysing solution. Flow cytometry results were compared in the same cases with immunocytochemistry results using the alkaline phosphatase anti-alkaline phosphatase method.

Results—The gentle permeabilisation induced by this method permitted preservation of the membrane antigens and the size and morphology of the cells. The results using flow cytometry were comparable with those obtained using immunocytochemistry, with nearly complete concordance in most cases.

Conclusions—This technique is simple, rapid, sensitive and reproducible and it is suitable for double staining procedures, such as nuclear and cytoplasmic, nuclear and membrane, or cytoplasmic and membrane. It therefore provides a powerful tool for extending the use of immunophenotyping for the diagnosis and follow up of acute leukaemia. It could also be used for the investigation of minimal residual disease.

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There has been considerable progress over the past few years in the characterisation of leukaemic cells by the use of cell markers in addition to morphological diagnosis. In acute leukaemia the findings have direct clinical and therapeutic relevance. The introduction of flow cytometry has improved the analysis of cell surface antigen expression.¹ Flow cytometry allows large numbers of cells (up to 1×10^6) to be analysed over a short period of time, and the possibility of quantitating the results makes this superior to immunofluorescence microscopy and immunocytochemistry. As several of the antigens detected in blast cells are expressed first in the cytoplasm, a

complete immunophenotype requires the detection not only of membrane antigens but also of intracellular ones.²⁻⁵

The most commonly used methods for detecting intracytoplasmic and nuclear antigens are immunofluorescence and immunocytochemistry on cytocentrifuge preparations of fixed cells. Although these microscopic techniques combine morphology with immunological information, they are time consuming, their results are somewhat subjective, and sometimes only a limited number of cells can be counted, giving inaccurate results. Flow cytometry for the detection of cytoplasmic and nuclear antigens has so far had limited use as the cells need to be permeabilised so that the monoclonal antibodies can reach the intracellular antigens. The reagents used for permeabilisation (fixatives and detergents) can also cause disruption of the cells with loss of antigen expression and distortion of the morphology and light scatter, so that it may not be possible to identify different cell populations.^{6,7}

We describe a technique that does not disrupt the cells and preserves both antigen expression and light scatter, and permits a precise study of the leukaemic cells by gating the desired population for analysis.

Methods

Peripheral blood and bone marrow anticoagulated samples from 40 patients with acute leukaemia at presentation or relapse were studied. The diagnosis was based on morphology and cytochemistry according to the FAB criteria.^{8,9}

Mononuclear cells were isolated by density gradient centrifugation with Lymphoprep (Nycomed SA, Oslo, Norway). Cells were washed twice with Hanks's balanced salt solution before being immunostained.

FIXATION AND PERMEABILISATION

Cells ($0.5-1 \times 10^6$) were used for each test. The cells were incubated for 10 minutes at room temperature with 2 ml of a mixture of 1 ml 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) (final concentration 2%) and 1 ml of a 1 in 10 dilution of Becton Dickinson's FACS lysing solution in distilled water. This is a diethylene glycol based solution that causes lysis of the red cells and mild permeabilisation of the white cells. The cells were centrifuged in an immunofuge for two minutes, the supernatant fluid discarded, and then washed with 2 ml of 0.5%

Table 1 Monoclonal antibodies used in this study

Monoclonal antibody	CD number	Amounts and dilutions	Source
T3-RD1	CD3	5 µl neat	Coulter Clone, Hialehah, Florida
UCTH1		5 µl neat	University College, London
J5-RD1	CD10	5 µl neat	Coulter Clone, Hialehah, Florida
MY7-RD1	CD13	5 µl neat	Coulter Clone, Hialehah, Florida
MCS-2		10 µl neat	Nichere Corp, Japan
B4-RD1	CD19	5 µl neat	Coulter Clone, Hialehah, Florida
Leu-12-PE		20 µl neat	Becton Dickinson, Mountain View, California
MY9-RD1	CD33	5 µl neat	Coulter Clone, Hialehah, Florida
OKB 22	CD22	5 µl neat	Ortho Diagnostic System, Raritan, New Jersey
IgM-FITC		50 µl 1 in 200	Dakopatts, Glostrup, Denmark
Anti-MPO		50 µl 1 in 10 ³	CBL, The Netherlands
MPO-1		5 µl neat	Dakopatts, England
MPO-7		30 µl 1 in 10	Seralab, Crawley Down, England
Rabbit anti-Tdt		30 µl 1 in 50	Seralab, Crawley Down, England
Swine anti-Goat anti-mouse-FITC		50 µl 1 in 200	Cappel, North Carolina, USA
Goat anti-mouse-PE		50 µl 1 in 20	Dakopatts, England
Mouse IgG-RD1		5 µl neat	Coulter Clone, Hialehah, Florida

FITC = fluorescein isothiocyanate; PE = phycoerythrin; RD = rhodamine.

Tween 20 in PBS in an immunofuge for two minutes.

INDIRECT IMMUNOFLOUORESCENCE STAINING

The fixed cells were incubated with the appropriate amount of each monoclonal antibody adequately titrated to find the best dilution (table 1) for 20 minutes at room temperature.

After two washes with 0.5% Tween 20 as above, the cells were incubated with fluorescein isothiocyanate (FITC)-conjugated second antibody (swine anti-rabbit or goat anti-mouse immunoglobulins) for 10 minutes at room temperature. The cells were then washed twice and resuspended in 500 µl of Isoton and were then ready for analysis by flow cytometry. Negative controls were always used by treating the cells in the same way as above but omitting the first antibody.

DIRECT IMMUNOFLOUORESCENCE STAINING

Antibodies directly labelled to FITC, rhodamine (RD1), or phycoerythrin (PE) were used to stain the cells in only one step followed by two washes with Tween 20.

DOUBLE LABELLING

Membrane and cytoplasmic or nuclear

The cells were first stained for membrane antigens by incubation with 50 µl of 2% AB serum and the required amount of the RD1 or PE labelled monoclonal antibody for 10 minutes at room temperature, washed once with PBS-azide-bovine serum albumin, then fixed, permeabilised and stained for cytoplasmic or nuclear antigens with FITC labelled antibodies, as described above.

Cytoplasmic and nuclear

The cells were first fixed and permeabilised and then stained for both antibodies together. Both antibodies used for double labelling were either directly labelled, such as CD19-RD1 and IgM-FITC (both were mouse antibodies), or of different species (rabbit and mouse) if one of them was not directly

labelled, such as rabbit anti-TdT (not labelled) and membrane mouse anti-CD10-RD1 or CD19-RD1.

Paraformaldehyde powder was obtained from Sigma (St Louis, Missouri). Polyoxyethylene(20)sorbitan monolaurate (Tween 20) was obtained from BDH (England). FACS lysing solution was obtained from Becton Dickinson (San José, California). Table 1 shows the antibodies used in this study.

All samples for flow cytometry were processed on a FACScan Flow cytometer (Becton Dickinson) using Lysis II software. The data were analysed after gating the leukaemic cells in the light scatter (forward scatter against side scatter) dot plot using the normal counterpart patterns as described by Carter.¹⁰

IMMUNOCYTOCHEMICAL STAINING

Cytocentrifuge slides of mononuclear cells were prepared, air dried, wrapped in foil and stored at -20°C until immunocytochemical labelling. This was performed using the alkaline phosphatase-anti-alkaline phosphatase (APAAP) technique, as described elsewhere.¹¹⁻¹³ Briefly, the slides were fixed in pure acetone for 10 minutes and air dried. Monoclonal antibodies optimally diluted were added and incubated for 30 minutes. Subsequently they were incubated for 30 minutes with rabbit anti-mouse immunoglobulins (Dakopatts) and for 45 minutes with mouse APAAP complexes (Dakopatts). The reaction was developed with a solution containing naphthol AS-MX phosphate (Sigma), levamisole, dimethylformamide (Merck, England) and Fast Red TR salt (Sigma) for 15 minutes. Slides were counterstained with haematoxylin-S and mounted with glycergel (Dakopatts). For TdT, an extra step was included by incubating for 30 minutes with mouse anti-rabbit immunoglobulins as second layer, followed by the rabbit anti-mouse immunoglobulins (Dakopatts).

Results

Based on morphology, FAB criteria,^{8,9} and immunophenotyping,¹³ the 40 cases were classified as follows: 13 (32.5%) acute lymphoblastic leukaemia (ALL), 19 (47.5%) acute myeloid leukaemia (AML), three (7.5%) chronic granulocytic leukaemia in blast crisis; five (12.5%) were classified as biphenotypic.

This method is a modification of a technique supplied by Drs A Orfao and JF San Miguel from Salamanca University (personal communication) that uses Becton Dickinson lysing solution and Tween 20 1% for permeabilisation without prefixation of the cells. With this method the light scatter of the cells was disrupted and we were not able to identify any cell population. Our initial experiments were carried out to optimise the conditions required for the detection of intracytoplasmic and nuclear antigens for cells in suspension. Different concentrations of Tween 20 as a

Figure 1 Effect of fixation and permeabilisation on cell scatter. A and B represent normal peripheral mononuclear cells and C and D represent a case of AML. A and C are untreated while B and D were fixed with PFA and permeabilised with Becton Dickinson FACS lysing solution and Tween 20. FSC = forward scatter; SSC = side scatter.

permeabilising agent and different concentrations of PFA as fixative were used (data not shown). Tween 20 in concentrations higher than 0.5% caused disruption of the scatter and so did PFA in concentrations of less than 2%. The use of Tween 20 alone in such low concentrations omitting Becton Dickinson lysing solution does not produce the required permeabilisation; therefore, we used both of them to attain full permeabilisation retaining a good scatter (fig 1). There was minimal reduction in the forward scatter (cell size) but the side scatter was unchanged, thus allowing different cell populations to be clearly recognised.

For optimal double fluorescence staining it is essential to preserve the expression of membrane antigens (figs 2 and 3). The membrane antigen expression (percentage of positive cells and fluorescence intensity) is preserved with both CD10 (stronger) and CD19 (weaker) in fig 2 and with CD19 in fig 3.

Preservation of fluorescence intensity is shown in fig 4 where there are two popula-

tions of CD3 positive cells; one showing weak expression is TdT positive (blasts), the other showing strong CD3 expression is TdT negative (normal T lymphocytes).

In a case of biphenotypic acute leukaemia (coexpression of B and myeloid lineages), the blasts were positive for both TdT and MPO (fig 5).

This technique was able to demonstrate that the expression of TdT in AML and ALL is not the same. It is significantly stronger in lymphoblasts compared with myeloblasts and intermediate in ALL expressing myeloid antigens (fig 6).

To ascertain the sensitivity of this technique for detecting intracytoplasmic and nuclear antigens we compared the results with those obtained by APAAP which was the standard immunocytochemistry technique used in our laboratory. APAAP results were read independently of the results by flow cytometry. Table 2 shows the percentages of positive cells for nuclear and cytoplasmic antigens by flow cytometry (F) and APAAP (P).

Figure 2 A case of ALL showing preservation of membrane antigen expression and fluorescence intensity. (A) represents the negative control; (B) represents double labelling of the cells for TdT and CD19; and (C) represents double labelling for TdT and CD10. The blasts were double positive for TdT and both CD19 and CD10 and the expression of CD10 is stronger than the expression of CD19. CON = control; RD1 = rhodamine; FITC = fluorescein isothiocyanate.

Figure 3 A case of Pre-B ALL. The cells were double labelled for CD19 (RD1) and cytoplasmic μ (FITC). Igm = Cytoplasmic μ .

The differences (if any) in the proportion of positive cells with both methods were minimal.

Table 3 compares the number of positive cases using both techniques. Using 10% as a cutoff for TdT and MPO, and 20% for the other antibodies, we found nearly complete concordance between flow cytometry and APAAP except for CD22 where differences were observed in cases 14, 34, and 38 which were negative with one technique and positive with the other.

All the cases which were CD13 positive by flow cytometry were also positive by APAAP; however, the number of positive cells was usually lower by flow cytometry. In two cases (16 and 17) CD13 was negative on the membrane but both the blasts cells had cytoplasmic expression with equivalent results by flow cytometry and APAAP.

Discussion

We developed a fixation and permeabilisation procedure for the detection of intracytoplasmic and nuclear antigens because of their importance in the immunophenotyping of

Figure 4 A case of biphenotypic acute leukaemia where the cells showed positivity for T (CD2, CD3), B (CD19, CD22) lymphoid markers, and myeloid markers (CD13 and anti-MPO) (data not shown). (B) shows double labelling for TdT and CD10. (C) TdT and cyt CD3 where the blasts are TdT + /cyt CD3 + while normal T lymphocytes are TdT - /cyt CD3 +.

Figure 5 Double labelling for TdT (FITC) and MPO (PE) in a case of biphenotypic acute leukaemia (B lymphoid and myeloid). MPO = myeloperoxidase; PE = phycoerythrin.

acute leukaemia in which some antigens are expressed in the cytoplasm at an earlier stage of differentiation than in the membrane.²⁻⁵ We used PFA, Tween 20, and Becton Dickinson FACS lysing solution in a combination that permits optimal permeabilisation with good preservation and little alteration of cell morphology and light scatter. The role of PFA in this technique is to stabilise the cell membrane and preserve intracellular antigens and the scatter properties of the cells.¹⁴ As PFA does not cause permeabilisation of the cells, we used Tween 20 and Becton Dickinson lysing solution for permeabilisation. Tween 20 is a mild non-ionic detergent that permeabilises cell membrane slowly and gently when used in a concentration that is well below the one that causes cell disruption.

Figure 6 Histograms of three different TdT positive cases of acute leukaemia. (A) a case of AML with weak TdT expression; (B) a case of ALL expressing aberrant myeloid antigens. The TdT intensity in this case is stronger than in (A) but weaker than (C) which represents a case of B-lineage ALL lacking myeloid antigens.

The detection of intracytoplasmic antigens by flow cytometry has been described by several investigators using different chemicals and detergents for fixation and permeabilisation (summarised in table 4). Landy *et al*²³ used formaldehyde and digitonin and noticed alteration of the scatter, but with our method there was a minimal decrease in the size of the cells (forward scatter) with no change in the side scatter, so preserving different populations.

Syrjälä *et al*²⁴ used only Becton Dickinson FACS lysing solution for fixation and permeabilisation for the detection of TdT, but when we compared this method with ours and APAAP it gave a lower percentage of positive cells (data not shown). Slaper-Cortenbach *et al*¹⁷ were not able to detect TdT positivity in AML (n = 12). In our study 11 of 19 (57.8%) cases of AML were TdT positive; similar findings were made by Hirata *et al*,¹⁸ who studied TdT by flow cytometry in 14 cases of AML and found that 57.1% were positive using a cutoff of 5%, and Krause *et al*,²⁶ who detected TdT by immunoperoxidase in 44% of AML.

Using this technique we were also able to demonstrate that TdT expression differs in different types of acute leukaemia, being stronger in ALL, moderate in ALL with expression of myeloid antigens, and weak in AML. This confirms the observation of Adriaansen *et al*,²⁷ using fluorescence microscopy, that the intensity of TdT staining was weaker in AML than in ALL, and that of Paietta *et al*,²⁰ who used flow cytometry, biochemical assay, and molecular analysis to prove this differential expression.

Van Der Schoot *et al*²⁵ studied the expression of myeloperoxidase in AML by flow cytometry using buffered formaldehyde acetone fixation, and observed false positive results if more than 20% mature myeloid cells were present in the sample, caused by the release of MPO from these cells. Our results showed a good agreement between flow cytometry and APAAP for the demonstration of MPO. Therefore, our technique seems reliable and reproducible. Compared with other techniques, such as microscopy, it is simpler and more rapid, it does not cause cell aggregation or alteration in the morphology, nor does it affect the intensity of fluorescence, and it permeabilises both cytoplasmic and nuclear membranes.

The potential applications of this technique are many:

- (1) The routine detection of cytoplasmic antigens, such as cytoplasmic μ for the diagnosis of pre-B ALL, or CD13 and MPO for the diagnosis of minimally differentiated AML (MO). Here we have confirmed our earlier observation⁴ that the expression of CD13 in the cytoplasm precedes its membrane expression.
- (2) Double fluorescence staining for nuclear TdT or cytoplasmic antigens and membrane antigens (the expression of which is not affected by this method) is useful for the detection and follow up of minimal residual disease in acute leukaemia after treatment.

Table 2 Positive cells (%) for cytoplasmic and nuclear antigens by flow cytometry (F) and APAAP (A)

Case No	Diagnosis	TdT		MPO		CD22		CD3		CD13		IgM	
		F	A	F	A	F	A	F	A	F	A	F	A
1	AML	8	6	97	96	Negative	Negative	Negative	Negative	NT	NT	Negative	Negative
2	MBC (CGL)	1	Negative	40	60	5	3	11	9	52	50	6	2
3	AML (M1)	Negative	Negative	81	80	1	1	1	4	26	69	1	2
4	AML	Negative	Negative	68	84	5	6	4	3	62	85	6	4
5	AML	12	11	54	55	Negative	1	7	4	67	90	9	5
6	AML	30	28	23	19	8	NT	8	NT	79	92	NT	NT
7	ALL	85	89	Negative	Negative	46	60	5	8	NT	NT	6	8
8	Biphenotypic AL (B and M)	52	62	81	88	2	1	1	1	NT	NT	1	1
9	ALL	72	87	Negative	Negative	63	90	8	3	25	NT	5	Negative
10	Biphenotypic AL (T and M)	68	77	40	70	11	9	81	90	53	98	1	1
11	AML (M5)	4	Negative	19	22	3	2	1	Negative	60	95	2	Negative
12	ALL	62	83	NT	NT	61	65	NT	NT	76	88	5	4
13	ALL	78	80	1	Negative	25	55	3	2	56	400	4	2
14	ALL	62	80	39	20	13	98	4	3	4	Negative	1	2
15	AML (M1)	39	42	59	68	4	2	3	3	25	80	2	4
16	ALL	96	90	3	Negative	59	33	NT	NT	92	97	20	59
17	Biphenotypic AL (T, B and M)	67	75	36	50	75	56	99	85	77	37	36	38
18	AML (M2)	29	24	70	74	1	3	5	11	35	67	6	5
19	CGL (BC)	61	77	15	12	Negative	10	5	11	NT	NT	Negative	1
20	ALL	60	88	7	Negative	74	80	2	4	1	Negative	1	5
21	AML	Negative	1	76	90	7	1	8	19	54	65	4	5
22	ALL	52	69	Negative	Negative	31	73	6	4	NT	NT	31	65
23	AML	3	8	NT	NT	5	7	15	12	NT	NT	5	9
24	AML (M4)	Negative	Negative	57	80	13	5	2	5	48	50	NT	NT
25	Biphenotypic AL (T and M)	18	31	23	10	Negative	Negative	NT	NT	NT	NT	NT	NT
26	AML	51	57	40	47	NT	NT	NT	NT	NT	NT	NT	NT
27	AML	40	41	95	98	NT	NT	NT	NT	NT	NT	NT	NT
28	ALL	75	95	3	2	NT	NT	NT	NT	NT	NT	NT	NT
29	ALL	92	95	1	Negative	NT	NT	NT	NT	NT	NT	NT	NT
30	ALL	98	100	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
31	Biphenotypic AL (B and M)	58	65	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
32	AML	23	31	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
33	AML	1.4	Negative	86	90	6	2	3	2	91	68	3	5
34	ALL	23	NT	10	NT	29	7	13	10	43	21	7	6
35	AML (M5b)	62	49	98	NT	Negative	Negative	1	5	93	73	7	2
36	AML (M2)	22	19	90	90	NT	NT	2	4	91	90	Negative	Negative
37	CGL (BC)	93	93	62	84	76	90	7	2	91	87	4	15
38	AML	22	25	4	5	10	25	NT	NT	56	65	9	3
39	AML	33	41	88	78	7	14	NT	NT	44	64	NT	NT
40	ALL	81	98	3	6	50	64	2	12	1	Negative	1	1

IGM = cytoplasmic μ ; CGL (BC) = Blastic crisis of chronic granulocytic leukaemia; NT = not tested.

Table 3 Comparison of number of positive cases tested by flow cytometry (F) and APAAP (A)

TdT† (n = 40)		MPO† (n = 34)		CD22* (n = 29)		CD3 (n = 26)		CD13 (n = 25)		Cytoplasmic IgM (n = 27)	
F	A	F	A	F	A	F	A	F	A	F	A
31	31	24	24	11	12	2	2	22	22	3	3

*Cases 14, 34, 38 were positive by one technique and negative by the other.

†Cutoff of positivity was 10% while in the others it was 20%.

Table 4 Published reports on cytoplasmic and nuclear antigen detection using flow cytometry

Reference	Technique		Comments
	Fixation	Permeabilisation	
Schroff, <i>et al</i> ¹⁵	PFA and ethanol	Lyssolecithin	Detects anti-intermediate filament antigen
Lakhampal, <i>et al</i> ¹⁶	PFA and ethanol		Double staining for membrane antigens and DNA
Slaper-Cortenbush, <i>et al</i> ¹⁷	BFA		Detects CD3, CD22, TdT and Cyt. μ
Hirata, <i>et al</i> ¹⁸	Formalin	Tween 20 and methanol	Detects TdT
Bardales, <i>et al</i> ¹⁹	PFA	Saponin	Detects TdT
Paeta, <i>et al</i> ²⁰	PFA		Detects TdT
Schmid, <i>et al</i> ²¹	PFA	Tween 20	Double staining for surface antigens and DNA, detection of cyt CD3 and μ
Drach, <i>et al</i> ²²	BFA	Saponin	Detects MPO, CD3, CD22
Landy, <i>et al</i> ²³	Formalin	Digitonin	Double staining for membrane and cytoplasmic antigens
Syrjälä, <i>et al</i> ²⁴		BD FACS lysing solution	Detection of TdT
Van der Schoot, <i>et al</i> ²⁵	BFA		Detects MPO

PFA = paraformaldehyde; BFA = buffered formaldehyde acetone.

(3) For the rapid diagnosis of biphenotypic acute leukaemia by being able to use the full range of reagents to diagnose such cases which may require more intensive treatment protocols.

We conclude that the technique described provides a powerful tool for improving the application of immunophenotyping to the diagnosis, follow up, and overall management of all types of acute leukaemia. We expect that nuclear and cytoplasmic staining by flow cytometry may replace in the near future the need for performing immunocytochemistry on fixed cells in many of its current applications in the immunophenotyping of leukaemic blast cells.

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