ORIGINAL ARTICLE

Detection of cyclin D1 in B cell lymphoproliferative disorders by flow cytometry

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Aims: To describe and revise a flow cytometric assay for evaluating cyclin D1 overexpression in B cell lymphoproliferative disorders (B-LPDs).

Methods: Cyclin D1 expression was evaluated in 11 healthy controls and 51 patients with B-LPD by flow cytometry using the 5D4 monoclonal antibody. In 25 cases, experiments were repeated up to four times with mononuclear cells (MNC) fixed in ethanol for 1–120 days to evaluate the consistency of cyclin D1 expression. Flow cytometry results were compared with fluorescence in situ hybridisation (FISH) for the t(11;14) translocation in 19 patients and with immunohistochemistry (IHC) using the DCS-6 monoclonal antibody in nine patients.

Results: A mean fluorescence intensity ratio (MFIR) of 4.8 was defined as the cut off point for positivity based on cyclin D1 expression in healthy controls (mean + 3 SD). Ten patients overexpressed cyclin D1 by flow cytometry. These included five of eight patients with mantle cell lymphoma, four of 19 with chronic lymphocytic leukaemia, and one with follicular lymphoma. MFIR in the repeat experiments differed less than 25% in 20 of 25 patients and in no cases did it cross the cut off point. There was a good correlation between cyclin D1 expression by flow cytometry and FISH for t(11;14) in 15 of 19 patients and six of nine had concordant results with flow cytometry, FISH, and IHC.

Conclusion: Cyclin D1 expression remains fairly stable once MNC are fixed in ethanol and the flow cytometric assay can be used for the routine screening of B-LPD. Further comparisons between flow cytometry, IHC, and FISH may be needed to ascertain the diagnostic value of the flow cytometric assay.

The translocation t(11;14)(q13;q32) and cyclin D1 overexpression are the hallmarks of mantle cell lymphoma (MCL).[1] Various studies have demonstrated the diagnostic relevance of cyclin D1 overexpression in B cell lymphoproliferative disorders (B-LPDs), essentially to establish or confirm a diagnosis of MCL.[2–4] However, most of the available techniques to detect cyclin D1 are time consuming, technically difficult, and not readily available in most laboratories.[5]

We have described a method for detecting cyclin D1 by flow cytometry in B-LPD using the 5D4 monoclonal antibody.[6] In our present study, we have revised the technology for detecting cyclin D1 to overcome certain limitations that precluded its routine application. In the previous method, three days were required to obtain results in most cases and tumour cell lines needed to be used as controls in parallel to define the cut off point for positivity.[6] Furthermore, the absence of a well defined and uniform acquisition window sometimes caused the misinterpretation of results.

> "Most of the available techniques to detect cyclin D1 are time consuming, technically difficult, and not readily available in most laboratories"

The aims of our study are: (1) to describe a modified protocol that would overcome the aforementioned limitations; (2) to demonstrate the consistency and reproducibility of the modified protocol; (3) to evaluate cyclin D1 expression in 51 cases of B-LPD and to compare the flow cytometry results with cyclin D1 expression assessed by immunohistochemistry (IHC) and t(11;14) by fluorescence in situ hybridisation (FISH) in a selected group of patients.

MATERIALS AND METHODS

Samples and controls

Peripheral blood samples from 11 normal healthy controls (six women and five men) were used as negative controls to define the cut off point for cyclin D1 positivity. Cyclin D1 expression was evaluated in peripheral blood (n = 48) and bone marrow (n = 5) samples from 51 patients with B-LPD. The surface expression of CD19, CD20, CD22, CD5, CD23, CD79b, FMC-7, CD10 and κ/λ was analysed by the standard whole blood lysis method. B cells (CD19+) comprised more than 50% of the lymphoid cells in all cases and greater than 70% in 42 cases. The scoring system for the diagnosis of chronic lymphocytic leukaemia (CLL) was applied as described previously.[7 8] The diagnosis was primarily based on cell morphology according to French American British proposals and immunophenotyping, and substantiated by histology and FISH, wherever available. The 51 cases included: 20 CLL (immunophenotypic scores 4/5), three atypical CLL (score 3), and 28 B cell lymphomas (B-NHL) (scores 0–2). This last category included nine patients with MCL documented by histology and/or t(11;14) and 19 patients who were grouped as follows: CD5 positive B-NHL (14 patients) and CD5 negative B-NHL (five patients). The 14 CD5 positive B-NHL cases (eight of which were CD23 negative) included three splenic lymphomas with villous lymphocytes (SLVL), two follicular lymphomas (FLs), and one each of lymphoplasmacytic lymphoma, B prolymphocytic leukaemia, and large cell lymphoma; in six patients, where histology was not available, a definite diagnosis could not be

Abbreviations: B-LPD, B cell lymphoproliferative disorder; B-NHL, B cell lymphoma; BSA, bovine serum albumin; CLL, chronic lymphocytic leukaemia; CSB, cyclin staining buffer; FISH, fluorescence in situ hybridisation; FITC, fluorescein isothiocyanate; FL, follicular lymphoma; IHC, immunohistochemistry; MFF, mean fluorescein isothiocyanate fluorescence; MFIR, mean fluorescence intensity ratio; MNC, mononuclear cells; MCL, mantle cell lymphoma; PBS, phosphate buffered saline; PI, propidium iodide; SLVL, splenic lymphomas with villous lymphocytes

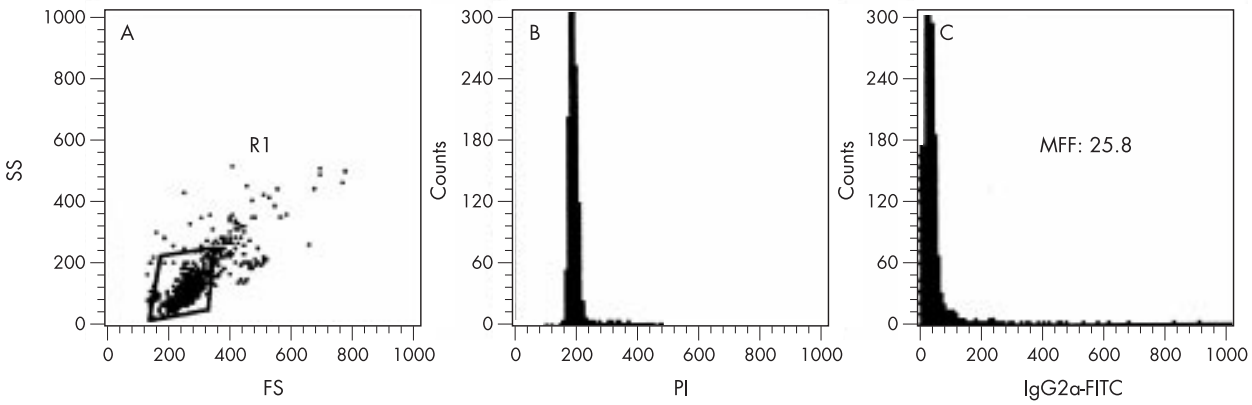

Figure 1 Acquisition window showing (A) light scattergram with forward scatter (FS) and side scatter (SS); (B) propidium iodide (PI) histogram plot with G0/G1 peak at ~ channel 200; (C) fluorescein isothiocyanate (FITC) histogram plot. During acquisition, the FITC voltage is regulated to reach a mean FITC fluorescence value between 20 and 30 with the isotype control. Appropriate compensation is done for spectral overlap of FITC and PI signals. Mononuclear cells stained with the anti-cyclin D1 monoclonal antibody are then acquired without changing the instrument settings.

made by morphology, immunophenotype, and/or FISH, and remained unclassified. Five cases of CD5 negative B-NHL included two SLVL, one FL, and two unclassified.

Antibodies and reagents

Fix and Perm fixation medium A (Caltag, Burlingame, California, USA), a formaldehyde based fixative.

70% ethanol in phosphate buffer saline (PBS; pH 7.3) stored at –20°C.

Cyclin staining buffer (CSB): PBS + 0.1% sodium azide (Sigma, Poole, Dorset, UK) + 1% bovine serum albumin (BSA; Sigma) + 0.1% Tween 20 (Sigma).

Anti-cyclin D1 unconjugated antibody: 5D4 (Coulter-Immunotech, Marseille, France) monoclonal antibody diluted 1/10 in PBS with 2% BSA. A 1 µl aliquot of the final solution contains 0.1 µg of 5D4 monoclonal antibody.

Anti-IgG2a unconjugated isotype control (Caltag): 1 µl contains 0.1 µg immunoglobulin.

Secondary antibody: fluorescein isothiocyanate (FITC) conjugated goat antimouse IgG $F(ab)_2$ fragment (ICN Biomedicals, Aurora, Ohio, USA) diluted 1/25 in PBS with 2% BSA.

Propidium iodide (PI; Sigma) 1% wt/vol stock solution diluted 1/20 in PBS.

Ribonuclease A (RNase; Sigma) 1% wt/vol stock solution stored at –20°C.

Immunostaining with cyclin D1

Cell separation

The mononuclear cells (MNC) were obtained by density gradient centrifugation (Histopaque 1077; Sigma). The cells were washed three times in Hanks balanced salt solution and resuspended at $1–2 \times 10^6$ cells in 100 µl of Hanks balanced salt solution in two tubes labelled "cyclin D1" and "control".

Fixation

A 70 µl aliquot of Fix and Perm solution A was added to both the tubes, which were gently vortexed and incubated at room temperature for 10 minutes. The cells were then washed once in PBS and 1.5 ml of 70% ethanol was added drop by drop to each tube. The tubes were then incubated at –20°C for one hour, centrifuged, and the supernatant discarded.

Permeabilisation

The cells were washed twice with CSB containing 0.1% Tween 20.

Primary antibody

A 5 µl aliquot of the anti-cyclin D1 and the anti-IgG2a isotype monoclonal antibodies was added to the respective tubes. The cells were gently vortexed, incubated overnight at 4°C, and washed twice with CSB.

Secondary antibody

The cells were incubated at room temperature in the dark for one hour with 50 µl of secondary monoclonal antibody. They were then washed twice in PBS and resuspended in 500 µl of isoton.

PI staining

RNase (15 µl) and 25 µl of PI were added 15 minutes before acquisition.

Flow cytometric analysis

Linearly amplified light scatter and fluorescent signals of 10 000 events were acquired using a FACSCalibur cytometer equipped with a 15 mW, 488 nm argon laser (Becton Dickinson, San Jose, California, USA). At acquisition, the FITC voltage was regulated to reach a mean FITC fluorescence (MFF) value between 20 and 30 with the isotype control. Appropriate compensation was done for spectral overlap of FITC and PI signals. MNC stained with the cyclin D1 monoclonal antibody were then acquired without changing the instrument settings (fig 1). The list mode data were analysed with Cell Quest software (Becton Dickinson). Doublets were excluded by gating on PI width and PI height dot plots. MFF values with the isotype and the anti-cyclin D1 monoclonal antibody were read from the respective FITC histogram statistics. Cyclin D1 expression was analysed as the mean fluorescence intensity ratio (MFIR). Therefore, MFIR = MFF with the anti-cyclin D1 antibody/MFF obtained with the isotype control.

Consistency and reproducibility

To evaluate the consistency and reproducibility of the results, MNC from 25 positive and negative cases (seven normals, six positives, and 12 negatives) were fixed in 70% ethanol and cyclin D1 expression was analysed twice in 12 cases, three times in 10 cases, and four times in three cases. In total, 41 repeat experiments were performed over a period of 120 days (1–7 days, 10 cases; 8–30 days, 18 cases; 31–60 days, eight cases; 61–120 days, five cases).

FISH and immunohistochemistry

FISH analysis for the t(11;14) translocation was carried out in 19 cases with commercial probes for cyclin D1 (11q13) and the

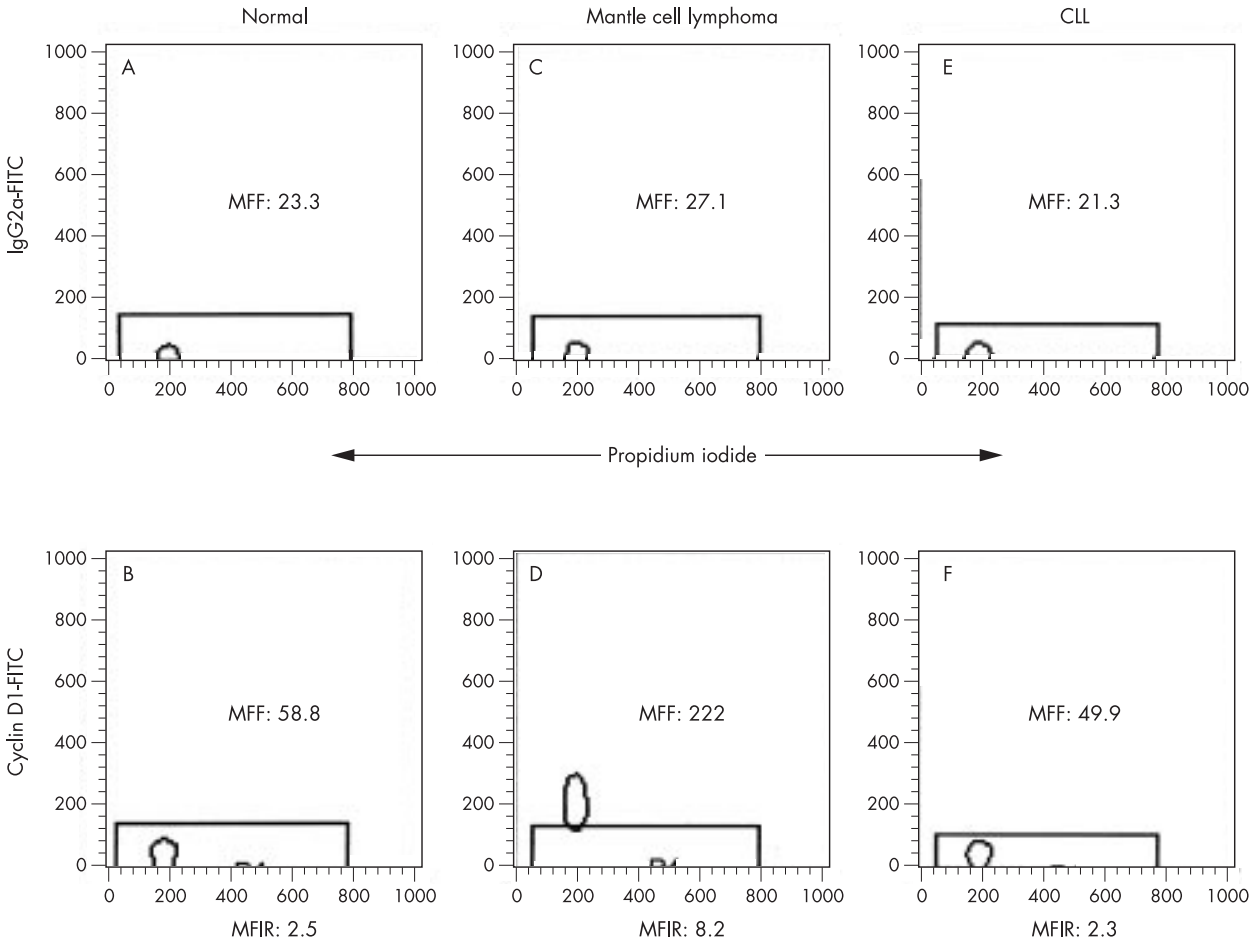

Figure 2 Representative contour plots illustrating cyclin D1 expression by flow cytometry in (A,B) a healthy normal control, (C,D) mantle cell lymphoma, and (E,F) chronic lymphocytic leukaemia. MFIR, mean fluorescent intensity ratio; MFF, mean fluorescein isothiocyanate (FITC) fluorescence.

IgH locus (14q32) (Vysis, London, UK). The probes were prepared according to the manufacturer's instructions and analysis was performed as described previously.[9] In nine cases, tissue sections from either lymph nodes or spleen were evaluated for cyclin D1 expression by standard immunohistochemistry with a streptavidin–biotin–peroxidase method using diaminobenzidine chromogen as the substrate. Sections were pretreated with a target retrieval solution (pH 9.9; Dako, Ely, Cambridgeshire, UK) for 25 minutes in a 800 W microwave oven and incubated for one hour at room temperature with the DCS-6 monoclonal antibody (Dako) diluted 1/200.

RESULTS

Cyclin D1 expression by flow cytometry (fig 2)

Based on the values of MFIR obtained in 11 normal healthy controls (mean, 3; SD, 0.6), a MFIR value of 4.8 (mean, + 3 SD) was defined as the cut off point for positivity. According to this, cells from 10 of 51 cases overexpressed cyclin D1 by flow cytometry (table 1). The positive cases comprised MCL (five of eight), one CD5 positive B-NHL (one of 14), and CLL (four of 19). In all the positive cases, cyclin D1 overexpression was restricted to the G1 phase of the cell cycle. The three cases with atypical CLL and the five cases of CD5 negative B-NHL were cyclin D1 negative.

In four samples (one MCL, peripheral blood and bone marrow; one CLL, peripheral blood; one CLL, bone marrow) analysis was inconclusive because the lymphoid gate was heavily contaminated by neutrophils as a result of poor separation during density gradient centrifugation. Alcohol fixation alters the light scatter of neutrophils rendering them indistinguishable from lymphoid cells on scattergram.

Consistency and reproducibility

To demonstrate the consistency of our results over a period of time, we repeated the experiments on the MNC fixed in alcohol for 1–120 days in 25 cases. In 20 of 25 cases, the MFIR in the repeat experiments differed less than 25% from the previous and/or subsequent MFIR values; in five cases, it differed from 25% to 50%. However, the final result was not affected

Table 1 Cyclin D1 expression by flow cytometry in 51 cases of B-LPD*

	Number of cases	Number of positives
CLL	19	4
Atypical CLL	3	0
Mantle cell lymphoma	8	5
CD5+ B-NHL	14	1†
CD5– B-NHL	5	0
Total	49	10

Mean fluorescence intensity ratio in the positive cases was as follows: CLL (5.6, 6.0, 6.0, 8.3), MCL (4.9, 6.0, 7.7, 8.2, 9.7), follicular lymphoma (5.9).
*Results were inconclusive in 2 cases; †follicular lymphoma.
B-NHL, B cell lymphoma; CLL, chronic lymphocytic leukaemia; MCL, mantle cell lymphoma.

Table 2 Correlation between cyclin D1 by flow cytometry and FISH

	FISH for t(11;14)	
Flow cytometry	Positive*	Negative
Positive	4	1†
Negative	3	11‡
Total	7	12

*7 MCL; †1 follicular lymphoma; ‡ 6 CD5+ B-NHL, 3 CLL, 1 atypical CLL, 1 CD5 negative B-NHL.
B-NHL, B cell lymphoma; CLL, chronic lymphocytic leukaemia; FISH, fluorescence in situ hybridisation; MCL, mantle cell lymphoma.

because the MFIR did not cross the cut off point on the repeat experiments.

Correlation with FISH and IHC

Fifteen of 19 cases showed a good correlation between cyclin D1 expression by flow cytometry and the presence or absence of the t(11;14) translocation by FISH (table 2). In six of nine cases there was agreement between flow cytometry, IHC, and FISH results (table 3). Eleven of 12 cases without evidence of t(11;14) by FISH were also negative by flow cytometry. These included three cases of "unclassified" CD5 positive B-NHL in which a diagnosis of MCL had been suspected on morphology and immunophenotyping; tissue histology was not available in these cases. In only one case, where FISH was negative, cyclin D1 was positive by flow cytometry but negative by IHC. The peripheral blood morphology and immunophenotype (CD5 positive, CD23 negative, CD10 negative) of this case was suggestive of MCL, but lymph node histology favoured follicular lymphoma.

Cells from two MCL cases, confirmed by cyclin D1 expression on splenic sections by IHC and t(11;14) by FISH, had been found to be negative for cyclin D1 by flow cytometry and were re-evaluated in this study for confirmation. The repeat flow cytometry was still negative for cyclin D1. However, at acquisition we noted higher than usual background fluorescence, which might have obscured the cyclin D1 expression and resulted in false negativity.

DISCUSSION

We have previously reported the value of immunostaining in cell suspensions for the detection of cyclin D1 in several cases of B-LPD and documented a 90% correlation between flow cytometry and cyclin D1 mRNA expression by reverse transcription polymerase chain reaction.[6] However, the previously described technique was tedious and not easily reproducible in routine practice. In our subsequent experience with the technique, we noted that sometimes results were misinterpreted and appeared inconsistent because of the absence of well defined settings at acquisition. Another disadvantage was that we used tumour cell lines to define the cut off for positivity, and as controls run in parallel with test samples. This requires a separate set up and expertise in maintaining cell lines, which is not readily available to most flow cytometry laboratories. In addition, it was a three day procedure usually requiring two overnight incubations, one in alcohol, and the other with the primary antibody.

In our present study, we regulated the FITC voltage during acquisition to keep the MFF with the isotype between channels 20 and 30. The uniform acquisition window thus achieved ensured comparability between samples with varying degrees of non-specific background fluorescence and enhanced the reproducibility of results. We have demonstrated the consistency of cyclin D1 expression in MNC fixed in alcohol from one hour to 120 days. This has several useful practical implications. First, by reducing the fixation time in alcohol to one hour, samples can be analysed in two days without compromising the accuracy of the results. Second, MNC fixed in alcohol at a peripheral hospital may be transported to a centre without the risk of loss of cyclin D1 expression. This is important, because whole blood/bone marrow samples more than 36–48 hours old tend to separate poorly by density gradient centrifugation and give inconsistent results for cyclin D1. Third, it implies that MNC fixed in alcohol from one experiment can be used as controls in the subsequent experiments, especially when using a new batch of antibody. In addition, we have further curtailed the dependence of the assay on cell lines by defining the cut off for positivity with normal healthy controls.

> "Concordance between flow cytometry, immunohistochemistry, and fluorescence in situ hybridisation was seen in 67% cases"

Flow cytometry for the detection of cyclins was originally developed in tumour cell lines to study the differential expression of cyclins with respect to the cell cycle.[10 11] Cyclin D1 expression is usually restricted to the G1 phase in MCL and cell lines with BCL-1 rearrangement.[6 12] Although staining with PI is not an essential prerequisite for measuring cyclin D1 overexpression in a diagnostic assay, it may sometimes reveal aberrant cyclin D1 expression in MCL with a complex karyotype.[13] In our study, 7.5% of samples were inconclusive because of neutrophil contamination on the lymphoid gate but this could be identified by an abnormal G0/G1 peak (fig 3). This pattern can be distinguished from an abnormal G0/G1 peak as a result of aneuploidy because the latter persists even with omission of the alcohol fixation step. An attempt to

Table 3 Correlation between cyclin D1 by flow cytometry, FISH, and immunohistochemistry

Diagnosis	Flow cytometry	FISH	Immunohistochemistry
MCL	Positive	Positive	Positive
MCL	Positive	Positive	Positive
MCL	Negative	Positive	Positive
MCL	Negative	Positive	Positive
CD5+B-NHL	Positive	Negative	Negative
CLL	Negative	Negative	Negative
CLL	Negative	Negative	Negative
CLL	Negative	Negative	Negative
Atypical CLL	Negative	Negative	Negative

The 5D4 clone anti-cyclin D1 antibody (Immunotech) was used for flow cytometry; the DCS-6 clone anti-cyclin D1 antibody (Dako) was used for immunohistochemistry.
CLL, chronic lymphocytic leukaemia; FISH, fluorescence in situ hybridisation; MCL, mantle cell lymphoma.

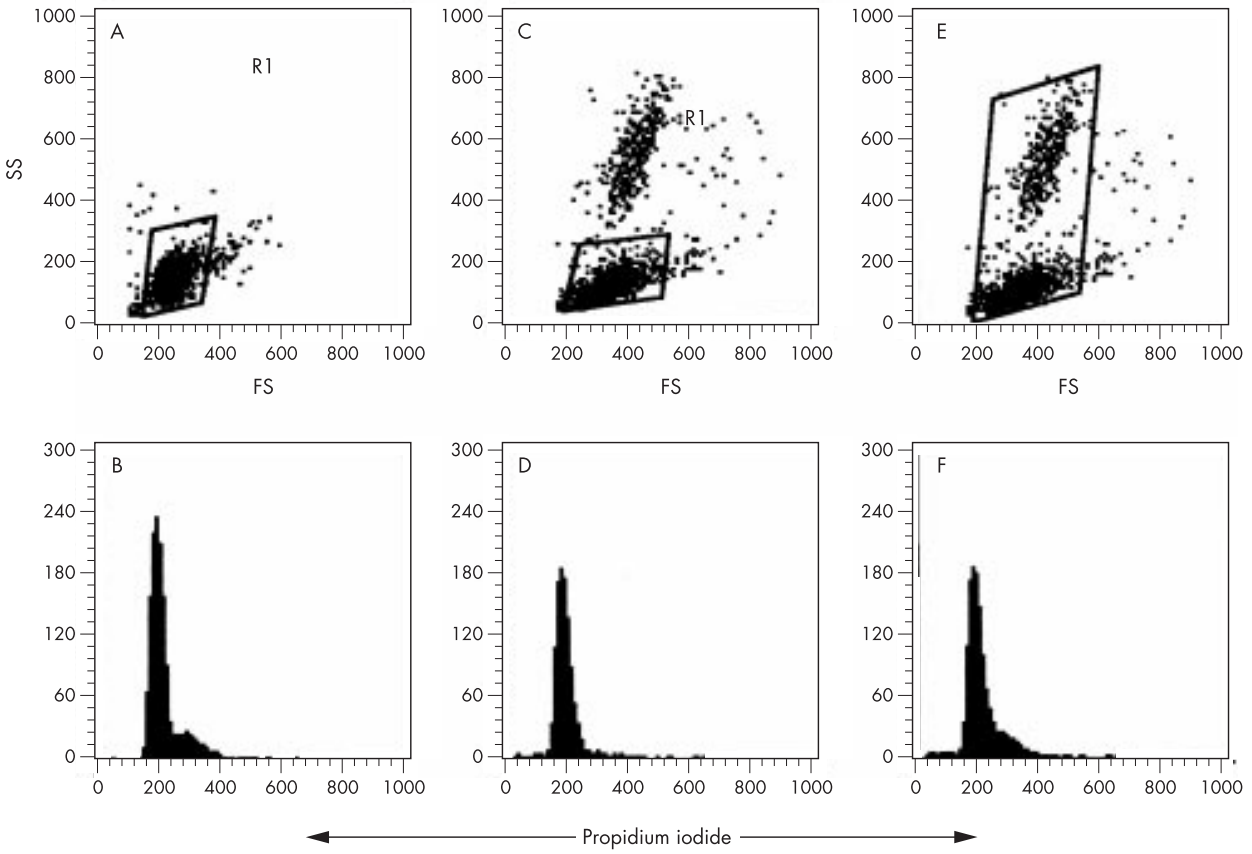

Figure 3 (A,B) Illustrate the effect of contamination of lymphoid gate by neutrophils as a result of alcohol fixation. (B) The propidium iodide (PI) histogram plot shows a widening of the G0/G1 peak. However, the scattergram (A) looks normal. (C,D,E,F) When the experiment was repeated with omission of the alcohol fixation step, the neutrophils separated from the lymphoid cells (C,E). Whereas gating around the lymphoid cells (C) revealed a normal G0/G1 peak (D), a gate including neutrophils (E) exhibited a G0/G1 pattern (F) similar to that seen in (B). Results on cyclin D1 expression with an abnormal G0/G1 pattern are often inconsistent and inconclusive. FS, forward scatter; SS, side scatter.

increase the purity of samples by surface CD19 staining was not successful because FITC conjugated antimouse secondary antibody also bound to CD19. Further manipulation by using a non-mouse CD19 monoclonal antibody (not available commercially at present) would be invaluable.

The monoclonal antibodies 5D4 and DCS-6 have been reported to detect 85–90% of MCLs by IHC.[14 15] However, DCS-6 is a more specific antibody because, unlike 5D4, it does not crossreact with cyclin D2. In this study, 62% of MCLs expressed cyclin D1 by flow cytometry. A negative case selection bias may be partly responsible for the apparently low sensitivity of our flow cytometry assay. However, this may also be the result of tissue based differences in cyclin D1 expression because IHC was performed on lymph node and spleen, whereas flow cytometry was carried out on peripheral blood and bone marrow. In contrast to tissue specimens, which are processed immediately in fixative, the delay in the fixation of peripheral blood samples as a result of transport may have caused some loss of cyclin D1 expression. Concordance between flow cytometry, IHC, and FISH was seen in 67% cases. However, only 18% of all cases were evaluated by all three techniques. Further studies are required to validate flow cytometry results with IHC on lymph nodes and/or peripheral blood cytospins, and the presence of the t(11;14) translocation as assessed by FISH.

Low level cyclin D1 expression has been reported to have prognostic importance in CLL[16] and atypical lymphoproliferative disorders.[17] In our study, 21% cases of CLL overexpressed 5D4 at levels comparable with MCL cases. Information regarding FISH and IHC was unfortunately not available in the positive cases. In a recent study, strong cytoplasmic staining for cyclin D1 was noted in 26% of CLLs using IHC with the monoclonal antibody DCS-6.[18] A distinction between cytoplasmic and nuclear staining is not possible in the flow cytometry assay and it is possible that the positive reaction that we saw in CLL cases was the result of cytoplasmic accumulation of cyclin D1. The cause and importance of this cytoplasmic accumulation remains to be investigated. It may be argued that 5D4 positivity in CLL was the result of its crossreactivity with cyclin D2, but then a greater proportion of CLL cases should have exhibited 5D4 positivity, because overexpression of cyclin D2 mRNA has been reported to occur in 85% of CLL cases.[19]

In conclusion, we have demonstrated the consistency of cyclin D1 expression in MNC fixed in ethanol and described a flow cytometry method to detect cyclin D1 expression in B-LPD, which, when compared with the previous technique, has well defined acquisition settings, does not depend on the use of tumour cell lines, and saves one day in sample processing. Larger studies comparing cyclin D1 expression by flow cytometry with IHC and the presence of the t(11;14) translocation are required to ascertain the value of this test in a diagnostic setting.

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Take home messages

- We have improved our earlier technique for detecting cyclin D1 expression in B cell lymphoproliferative disorders
- This improved technique has well defined acquisition settings, does not depend on the use of tumour cell lines, and saves one day in sample processing
- Cyclin D1 was expressed consistently in mononuclear cells fixed in alcohol for one to 120 days. This is useful because it means that the fixation time for the assay can be reduced, cells can be transported between laboratories, and the same batch of cells can be used in several different assays
- Larger studies comparing cyclin D1 expression by flow cytometry with immunohistochemistry and the presence of the t(11;14) translocation are required to ascertain the value of this test in a diagnostic setting

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