

Intracellular antigen determination by flow cytometry in leukaemic cells

SF CHIN, BSc (Hons.) and SK CHEONG, FRCP, FRCPA

Haematology Unit, Department of Pathology, Faculty of Medicine, Universiti Kebangsaan Malaysia.

Abstract

Several fixation and permeabilization techniques that enable the flow cytometric analysis of the cell contents have been introduced in recent years. These methods allow sensitive detection of intracellular antigens that facilitates the diagnosis of certain diseases. We have undertaken in this study to evaluate a simple method of fixation and permeabilization using 2% paraformaldehyde and Tween 20. Intracellular antigens in three different leukaemia cases were analysed. We found that the method was reliable and easy. Intracellular kappa light chains were found in abundance in a case of plasma cell leukaemia. CD3 and CD22 were found in greater amount intracellularly than on the surface in pre-T-ALL and pre-pre B-ALL respectively.

Key words: fixation, permeabilization, cytoplasmic antigens.

INTRODUCTION

The study of cell surface antigen expression has been facilitated by the availability of bench-top flow cytometers, highly specific monoclonal antibodies and immunofluorescent staining techniques. However, flow cytometric analysis have been restricted to the study of cell surface antigens because the fixation and permeabilization steps involved in the study of cytoplasmic antigens cause morphological damages, cell aggregation and loss of intracellular antigenicity.¹ Recent advances in the permeabilization procedures have enabled more in-depth studies of intracellular antigens. Certain antigens such as CD3^{2,3} and CD22⁴ can be detected intracellularly in early T and B-cell progenitors respectively. The ability to permeabilize the membrane and the availability of monoclonal antibodies (Ki-67⁵ & PC10⁶) that stain nuclear antigens in proliferation have also paved the way for a safe method of cell cycle analysis by flow cytometry. Many methods of fixation and permeabilization that allow cytoplasmic antigens analysis have been developed and in this study, the method used was based on the study by Schmid *et al*, 1991.⁷

MATERIALS AND METHODS

Blood samples

Venous blood from three leukaemic patients were collected in ethylenediaminetetraacetic acid (EDTA) anticoagulated vacutainer tubes (Becton Dickinson, USA). The blood was processed within six hours of collection.

Preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMC) were isolated from whole blood by gradient centrifugation on lymphocyte separation medium (Lymphoprep, Nycomed Pharma AS, Norway; density 1.077 g/ml). The extracted lymphocytes were washed twice by centrifuging at 250g for five minutes in phosphate buffered saline (PBS) pH 7.4. Cells that would be stained with the kappa/lambda monoclonal antibodies had to be washed a few more times to rid the cells of plasma immunoglobulins. The cells were resuspended in an appropriate volume of PBS.

Fixation

A hundred microlitre of the above cell suspension was dispensed into 12 x 75 mm Falcon tubes (Becton Dickinson). The cells in each tube was pelleted by centrifuging at 250g for 5 minutes. The pellet was resuspended in 875 μ l of cold PBS and 125 μ l of cold 2% paraformaldehyde (Sigma, USA) in PBS (pH 7.2). The mixture was vortexed immediately and incubated at 4°C for one hour. The mixture was centrifuged at 250g for five minutes and the pellet was retained.

Permeabilization

The fixed cells were resuspended in one ml 0.2% polyoxyethylenesorbitan laureate (Tween 20; Sigma) in PBS at room temperature. The suspension was gently mixed. Following incubation for 15 minutes at 37°C, one ml of PBS supplemented with 2% foetal calf serum (FCS;

Gibco) and 0.1% sodium azide (NaN_3 ; BDH) was added. The mixture was centrifuged at 250g for 5 minutes and the cell pellet retained.

Staining

All the three samples were analysed for cell surface markers routinely used for the diagnosis of leukaemia in this laboratory i.e. CD2, CD3, CD4, CD5, CD7, CD8, CD10, CD11c, CD14, CD16, CD19, CD22, CD25, CD33, CD34, CD45, CD56, CD61, glycophorin A, HLA-DR and kappa & lambda light chains.

For sample 1, which was obtained from a plasma cell leukaemia patient, the cytoplasmic contents of both kappa and lambda light chains were determined using monoclonal antibodies obtained from Becton Dickinson. Sample 2 was from a patient diagnosed with T-Acute Lymphoblastic Leukaemia (T-ALL). Its cytoplasmic content of CD3 was determined with anti-CD3/CD19 (Simultest CD3/CD19, Becton Dickinson). Sample 3 was obtained from a patient with pre-pre-B ALL and was stained with CD22 and kappa/lambda light chains. Mouse IgG isotypic controls (Becton Dickinson) were used as negative controls. The cell pellet in each tube was resuspended in 100 μl of PBS with 2% FCS and 0.1% NaN_3 . The cell suspension was incubated at 4°C for 30 minutes with the respective monoclonal antibodies. The suspensions were then washed twice by centrifuging in PBS containing 0.2% Tween 20 at 250g for five minutes.

Flow cytometric analysis

A total of 6000 cells were acquired for each staining using the FACScan Research software and analysed with the LYSYS program in the FACScan flow cytometer (Becton Dickinson). The lymphocytes were gated according to their light scattering characteristics i.e. forward and right angle scattering. The cursors for the quadrants were set using the mouse IgG isotypic controls so that less than 2% of the cells were positive.

RESULTS

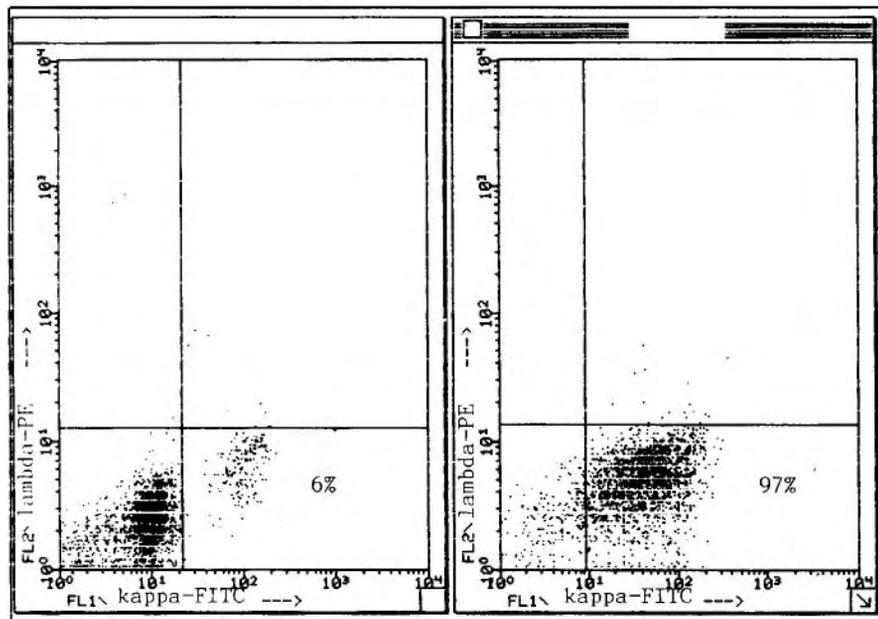
In patient 1, only 6% of the cells were staining for surface immunoglobulins (kappa light chains) but after treating the cells with paraformaldehyde and Tween 20, we found that 97% of the cells were staining intensely with anti-kappa light chain but not with anti-lambda chain (Fig. 1). We found that less than 8% of the cells from

patient 2 were staining for anti-CD3. After fixation and permeabilization, 85% of the cells demonstrated the presence of intracytoplasmic CD3 but not CD19 (Fig. 2). In patient 3, 50% of the cells were dimly positive for CD22 on the surface and the percentage of positive cells almost doubled after treatment (Fig. 3).

DISCUSSION

The results obtained in this study showed that the method of fixation and permeabilization using paraformaldehyde and Tween-20 is a useful procedure for allowing the quantitation of intracellular antigens both in the cytoplasm and the nucleus. The reagents employed in this method were readily available in any flow cytometry laboratory. The method was easy and did not require any special equipment and expertise except for flow cytometric analysis. The light scatter characteristics of the mononuclear cells were preserved thus allowing easy recognition and gating of the various leucocyte population. The threshold for forward scatter has to be reduced to accommodate for cells which have been shrunken after fixation with paraformaldehyde. We found that the pH of the 2% paraformaldehyde solution was critical for a slight pH variation was found to cause a drastic change in the light scatter characteristics of the fixed cells where the cells were noted to have a much lower forward scatter and side scatter (Fig. 4). Therefore, we suggest that the 2% paraformaldehyde be prepared fresh or the pH of stored solution should be checked each time before use.

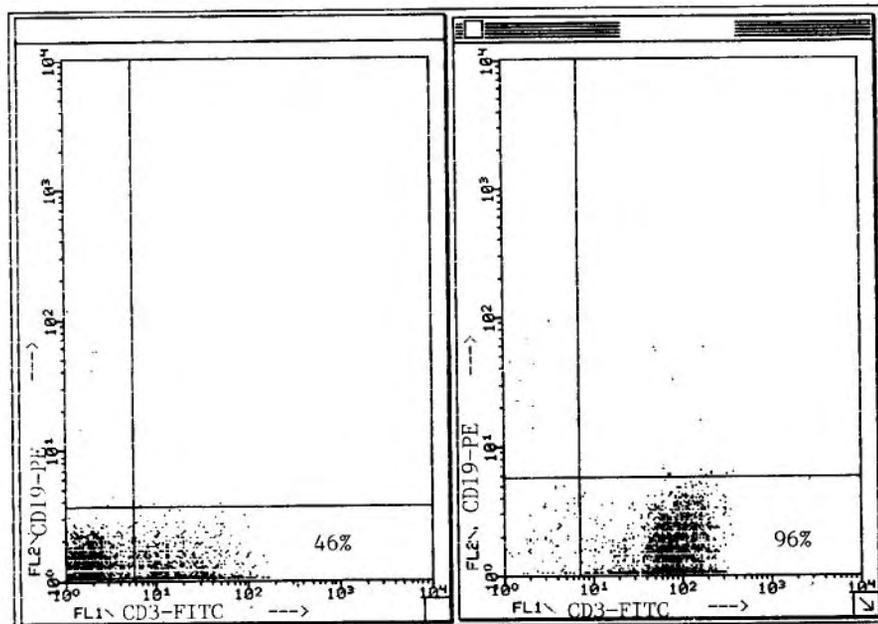
Plasma cell leukaemia is a rare disease caused by the neoplastic proliferation of a B cell at the late stage of the B lymphoid differentiation pathway. Plasma cells have been found to be unreactive with most of the pan-B markers except CD38 and cytoplasmic immunoglobulins (Ig).⁸ Since plasma cells are the production unit of immunoglobulins and when these immunoglobulins were vastly being produced but not secreted, then the immunoglobulin would be found in abundance within the cells. By permitting the entry of fluorescent antibody probes for the immunoglobulin light chains into the cells, then the presence of the cytoplasmic immunoglobulins could be detected as was shown in case 1. A common observation in B-chronic lymphocytic leukaemia (B-CLL) cases is that the cells have very low levels of Ig but it is found in abundance intracellularly.^{9,10} Therefore, the determination of cytoplasmic immunoglobulins



(a)

(b)

FIG. 1: Immunotyping results using anti-kappa/anti-lambda light chain monoclonal antibodies in a plasma cell leukaemia patient.
 (a) before fixation and permeabilization
 (b) after fixation and permeabilization.



(a)

(b)

FIG. 2: Immunotyping results using anti-CD3/anti-CD19 monoclonal antibodies in a pre-T-ALL patient
 (a) before fixation and permeabilization
 (b) after fixation and permeabilization.

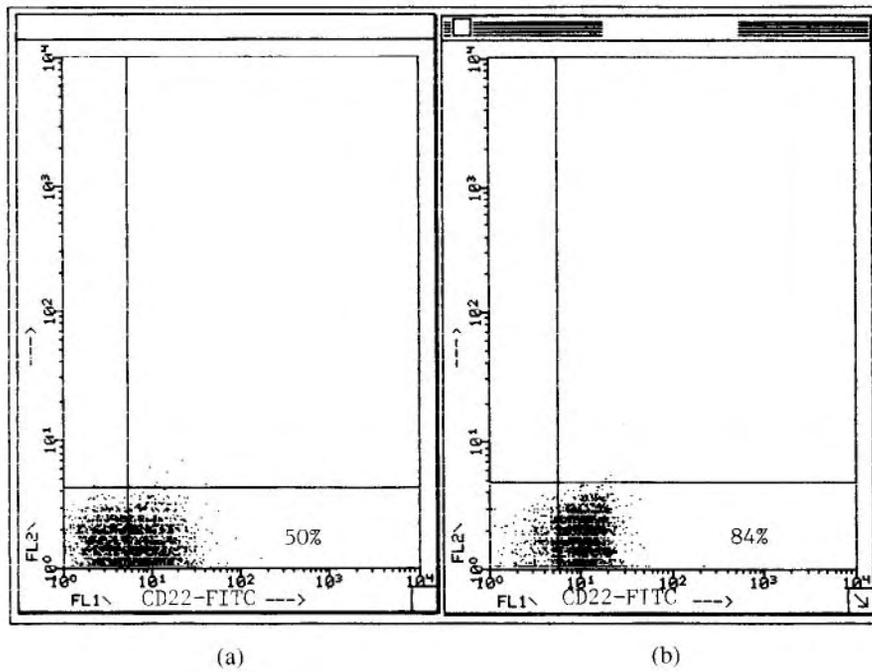


FIG. 3: Immunotyping results using anti-CD22 monoclonal antibody in a patient with pre-B-ALL
(a) before fixation and permeabilization
(b) after fixation and permeabilization.

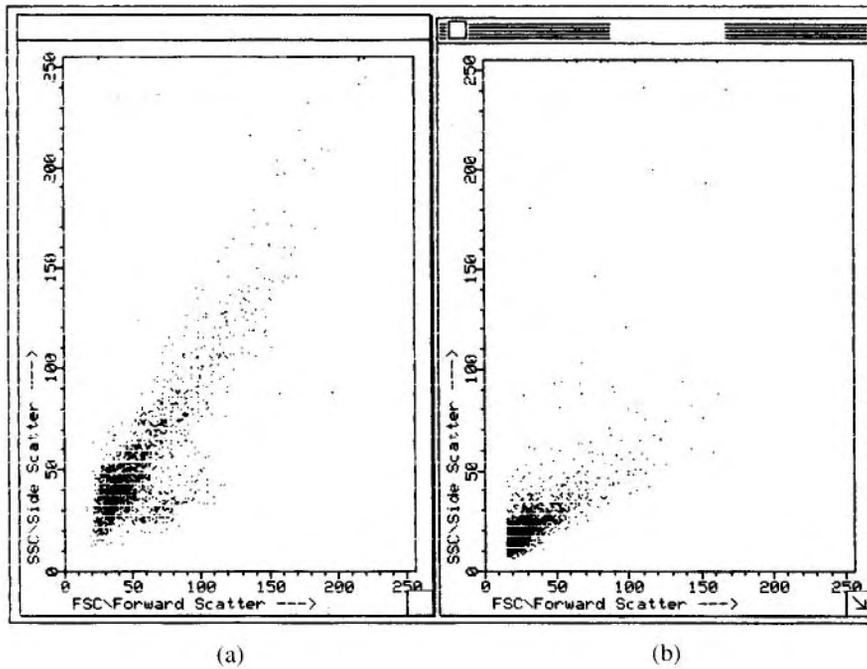


FIG. 4: The light scatter characteristics of mononuclear cells fixed with 2% paraformaldehyde with different pH
(a) pH 7.2
(b) pH 7.5.

is more useful for immunotyping of non-secretory plasma cell dysplasias and B-CLL.

The detection and quantitation of the percentage of cells with certain antigens that are expressed intracellularly at different stages of maturation such as CD3^{2,3} and CD22⁴ allows accurate classification of the cells. As in our case of the T-ALL where certain T-cell antigens (CD2, CD7 & CD8) were detected on the surface of the blast cells but not CD3 which is a sensitive pan T cell antigen, we found that more than 85% of the blasts were positive for intracellular CD3. Since cytoplasmic CD3 has been proven as an important diagnostic marker for immature T cell malignancies,¹ we concluded that our case was a precursor T-ALL.

The ability to probe for intracellular CD22⁴ and IgM heavy chains" facilitates the diagnosis of pre-pre-B ALL cases. Our case of pre-pre B-ALL was positive for all the B markers tested except CD10. We found that not only the number of cells that expressed CD22 intracellularly were higher than those expressing surface CD22 but also the mean fluorescence intensity of CD22 expression was higher intracellularly.

The ability to study intracellular antigens has opened a whole new dimension in immunophenotyping. Surface antigens no doubt play an important role in facilitating the classification of the different types of leukaemia but determination of nuclear and other intracellular antigens also facilitate detection of residual disease. For example, terminal deoxynucleotidyl transferase (TdT) is an enzyme that functions as a nuclear marker for acute lymphoblastic leukaemia^{12,13,14} and as an indicator for residual leukaemic cell content during and after chemotherapy. By applying a technique that allows simultaneous quantitation of both intracellular antigens e.g. TdT and surface antigens e.g. CD10, a better detection system of minimal residual disease after therapy can be established.

ACKNOWLEDGEMENT

This project was partly funded by IRPA grant No: 03-07-03-059.

REFERENCES

1. Andersson U, Halldén G, Persson U, Hed J, Möller G, DeLey M. Enumeration of IFN- τ -producing cells by flow cytometry: comparison with fluorescence microscopy. *J Immunol Methods* 1988; 112: 139-42.
2. Van Dongen JJM, Hooijkaas H, Comans-Bitter M. *et al.* Human bone marrow cells positive for terminal deoxynucleotidyl transferase (TdT), HLA-DR and a T cell marker may represent prothymocytes. *J Immunol* 1985; 135: 3144-50.
3. Link MP, Stewart SJ, Warnke RA, Levy R. Discordance between surface and cytoplasmic expression of the Leu-4 (T3) antigen in thymocytes and in blast cells from childhood T lymphoblastic malignancies. *J Clin Invest* 1985; 76: 248-53.
4. Dorken B, Pezzutto A, Köhler M, Thiel E, Hunstein W. Expression of cytoplasmic CD22 in B cell ontogeny. In: McMichael AJ, *et al* (eds): *Leucocyte Typing III, White Cell Differentiation Antigens*. Oxford University Press, England, 1987; p474.
5. Gerdes J, Schwab U, Lemke H, Stein H. Production of a monoclonal antibody reactive with human nuclear antigen associated with cell proliferation. *Int J Cancer* 1983; 31: 13-20.
6. Takasaki Y, Fishwild D, Tan EM. Characterization of proliferating cell nuclear antigen recognized by autoantibodies in Lupus sera. *J Exp Med* 1984; 159: 981-2.
7. Schmid I, Uittenbogaart CH, Giorgi JV. A gentle fixation and permeabilization method for combined cell surface and intracellular staining with improved precision in DNA quantification. *Cytometry* 1991; 12: 279-85.
8. Matutes E, Worner I, Sainati L, *et al.* Advances in the lymphoproliferative disorders. Review of our experience in the study of over 1000 cases. *Biol Clin Hematol* 1989; 11: 53-62.
9. Van der Reijden HJ, Van der Gaag R, Pinkster J, *et al.* Chronic lymphocytic leukaemia. Immunological markers and functional properties of the leukaemic cells. *Cancer* 1982; 50: 2826-33.
10. Pianezze G, Gentilini I, Casini M, *et al.* Cytoplasmic immunoglobulins in chronic lymphocytic leukaemia B cells. *Blood* 1987; 69: 1011-4.
11. Slaper-Cortenbach ICM, Admiraal LG, Kerr JM, *et al.* Flow cytometric detection of terminal deoxynucleotidyl transferase and other intracellular antigens in combination with membrane antigens in acute lymphatic leukaemias. *Blood* 1988; 72(5): 1639-44.
12. Kung PC, Long JC, McCaffrey RP, *et al.* Terminal deoxynucleotidyl transferase in the diagnosis of leukaemia and malignant lymphoma. *Am J Med* 1978; 64: 788-94.
13. Hutton JJ, Coleman MS, Keneklis TP, Bollum FJ. Terminal deoxynucleotidyl transferase as a tumour cell marker in leukaemia and lymphoma: results from 1000 patients. In: Fox M (ed). *Advances in medical oncology research and education. Biological basis for cancer diagnosis*. Pergamon Press, Oxford, 1979; 165-75.
14. Hetherington ML, Huntsman PR, Smith RG, Buchanan GR. Terminal deoxynucleotidyl transferase (TdT) containing peripheral blood nonnuclear cells during remission of acute lymphoblastic leukaemia: Low sensitivity and specificity prevent accurate prediction of relapse. *Leuk Res* 1987; 11: 537-43.