

Evaluating in-house anti-serum against B cells with flow cytometry

Suet-Feung CHIN, BSc. (Hons), Soon-Keng CHEONG, FRCP, FRCPA, Yaw-Chyn LIM, MSc, Kok-Lang MOK, BSc. (Hons) and Hussin Noor HAMIDAH, MBChB. DCP.

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

Abstract

The applications of antibodies, be it monoclonal or polyclonal, in the diagnostic and research fields are well established. The disadvantage is the high cost of commercially available antibodies. In a diagnostic establishment like ours which also functions as a training ground for laboratory related personnel, it is beneficial to be able to produce in-house reagents. Therefore, we have undertaken this project to produce a rabbit polyclonal antibody against B lymphocytes. We found that the rabbit was a good choice because the titre of antibody produced was high and positive reactions were still detected at a dilution of 1:38400. The antibody showed significant positive reaction only with the lymphocyte subpopulation. A positive reaction was observed between the immunized rabbit serum and B lymphocytes but not T lymphocytes. This shows that the antibody was B lymphocyte specific. There was a positive correlation between the percentage of B lymphocytes labelled using the commercial anti-CD19 monoclonal antibody and the in-house polyclonal antibody ($n=13$, $r=0.7$, $p=0.02$). However, the percentage of cells labelled by the in-house polyclonal anti-B was lower than that by the commercial monoclonal anti-CD19. The fluorescence intensity of the polyclonal antibody was lower than that of the monoclonal. In general, the performance of the in-house polyclonal antibody can be considered as satisfactory. The rabbit serum was stored at -20°C and no significant loss of activity was detected for over a period of 19 months.

Key words: Polyclonal antibody, B lymphocytes, phenotyping, flow cytometry.

INTRODUCTION

The extraordinary discriminatory power of antibodies especially monoclonal antibodies, has made it an indispensable tool for both diagnostic purposes and basic research. The main disadvantage is that commercial monoclonal antibodies are expensive. In a laboratory that also assumes a role in training personnel, the high cost may reduce the hands-on opportunity of the trainees. Thus, an alternate source of inexpensive anti serum would be beneficial. In this paper, we describe our experience in producing polyclonal antibodies from rabbits for immunochemical study, determining its titres and testing its specificity with flow cytometry. Flow cytometric immunophenotyping categorizes individual cells labelled with fluorochrome conjugated antibodies according to their size (degree of forward light scattering), internal complexity (degree of side light scattering), fluorochrome and fluorescence intensity.¹⁻⁵ The availability of the flow cytometer has allowed the determination of the distribution of immunoregulatory cells in the peripheral blood

and bone marrow aspirates in normal individuals and leukaemic patients⁷⁻⁸ and diseases that involve alterations in the lymphocyte subpopulations.⁹⁻¹⁰

MATERIALS AND METHODS

Preparation of immunogens

Polyclonal antibodies were raised against human B lymphocytes in rabbits. The cells were isolated from a patient with B-chronic lymphocytic leukaemia (B-CLL) using the Ficoll-hypaque gradient density centrifugation method.¹¹ The isolated cells were washed in RPMI containing 10% foetal calf serum (FCS). A cell count was obtained with the Coulter Jr. The cells were resuspended in freezing medium containing 20% FCS and 10% DMSO.¹⁴ Two millilitre of the above suspension (cell density of about 10^7 cells) were aliquoted into vials. The vials are then kept in liquid nitrogen (vapour phase) overnight and then transferred to the liquid phase for long term storage. For immunisation, one vial of stored cells was used.

The cells were brought rapidly to 4°C by warming the vial in a 37°C water bath until the ice had just melted. The cell suspension was washed repeatedly in RPMI and phosphate buffered saline (PBS) by centrifugation at 250g for five minutes to remove the DMSO.¹⁴ The cells were finally suspended in one millilitre of injection saline. The cells were tested for viability using Trypan Blue staining. To obtain a pure suspension of B lymphocytes, Dynabeads Pan-T (CD2) was used to remove residual T cells that may have been collected during the isolation procedure.

Animal rind Immunisation

The animal of choice was a male rabbit. The rabbit was bled from the marginal ear vein prior to immunisation to obtain normal rabbit serum to establish a baseline value. The baseline serum were aliquoted (0.5ml/vial) and stored at -20°C. Immunizations intravenously with purified H cells without adjuvant at an interval of two weeks were carried out. A test bleed from the marginal ear vein was taken before each subsequent injection to assess the rabbit's response. A booster was given to the rabbit when the titre of antibodies produced was high and the rabbit was sacrificed 7-10 days later."

Screening for antibody production

The serum collected from the rabbit (baseline/immunized) was diluted in serial doubling dilutions from 1:150 to 1:38400. The diluted serum was then used to label leucocytes from a normal individual.

An appropriate amount of whole blood was lysed with ammonium chloride (NH₄Cl) (one volume of whole blood to 20 volume of NH₄Cl).¹¹ The above suspension was incubated for 10 minutes at room temperature (KT). The cells were then washed three times by centrifuging in PRS for five minutes at 250g. The pelleted leucocytes were resuspended in 0.5 - 1.0 ml of PRS.

A three step immunolabelling method was employed i.e.:

First layer: Test/baseline rabbit serum (30 mins / RT)

Second layer: Mouse anti-rabbit immunoglobulin (30 mins / RT)
(Dako, Japan)

Third layer: Goat anti-mouse immunoglobulin conjugated with FITC (20 mins / KT / dark)
(Becton Dickinson, USA)

The cells have to be washed after each step.^{11,16} After the final wash, the cells are fixed in 1% paraformaldehyde. A total of 15000-20000 labelled cells were acquired with the FACScan Reserch software in the FACScan (Becton Dickinson, USA) within 24 hours of preparation and the data were analysed with the LYSYS software. The presence of the antibody was detected by the presence of positive fluorescence reaction as compared to the baseline serum. The cursors for the quadrant were set using the baseline control so that less than 2% of cells were positive. The antibody titre can be obtained by determining the highest dilution at which a positive result remains demonstrable.

Reaction with different leucocyte subsets

The treated cells (leucocytes from a normal individual incubated with rabbit serum) that were acquired can be grouped into four different regions according to their light scatter characteristics (forward and side scatter) as shown in Table 1. A pictorial example of the zoning performed is as shown in Fig. 1.

Cross-reactivity with T lymphocytes

Leucocytes from a normal individual was extracted with the above procedure. T lymphocytes were extracted using CD2 coated Dynabeads (Dynatcch, USA). The purity of the extracted T lymphocytes was tested by incubating the cells with fluorochrome conjugated anti-CD3 and anti-CD19. The stained cells were acquired and analysed. Cross-reactivity between the antiserum and T lymphocytes was tested by incubating both the cells and the antiserum (baseline/immunized rabbit serum) together. Only the lymphocyte population was analysed.

TABLE 1: The light scatter characteristics of leucocytes

Region	Leucocyte subsets	FSC	SSC
Region 1 (R1)	Lymphocytes	I	L
Region 2 (R2)	Granular lymphocytes & monocytes	H	I
Region 3 (R3)	Granulocytes	H	H
Region 4 (R4)	Red blood cells/debris	L	L

FSC = forward scatter; SSC = side scatter.

H = high amount of scattering

I = intermediate amount of scattering

L = low amount of scattering

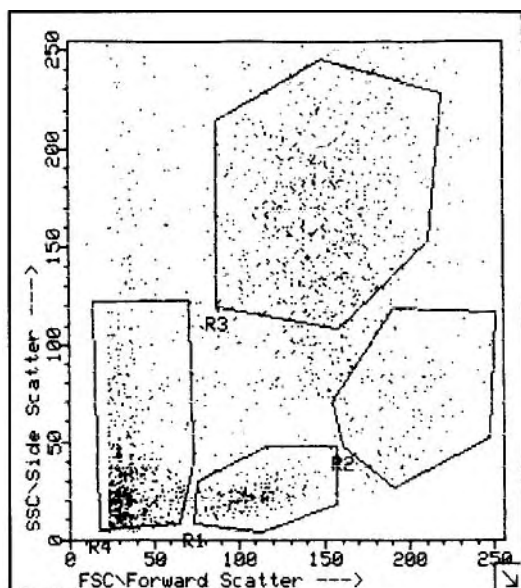


FIG. 1: Zoning of leucocytes based on their light scatter characteristics.

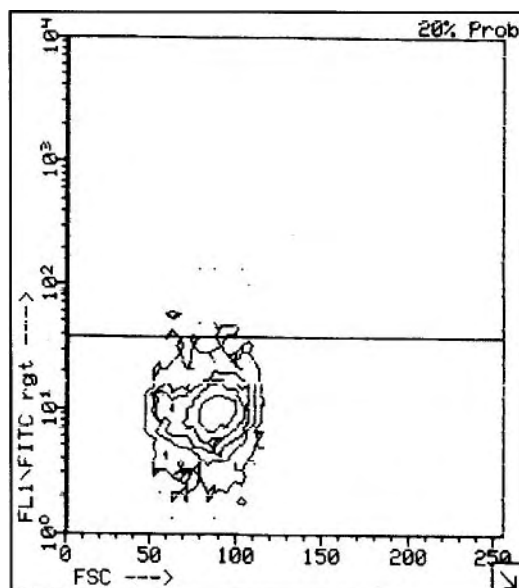


FIG. 2: Immunolabelling of T cells with the in-house polyclonal antibody.

Cross-reactivity with various types of leukaemic cells

Leucocytes were extracted from thirteen individuals diagnosed to have B Acute Lymphoblastic Leukaemia, Acute Myeloid Leukaemia, T-Suppressor Acute Lymphoblastic Leukaemia, Chronic Myeloid Leukaemia with blast transformation and B Chronic Lymphocytic Leukaemia. These cells were treated with the three step immunolabelling procedure as above. The antiserum was also tested with cells that were used as the immunogen. Only the lymphocyte population was studied. A comparison between the reaction with the in-house antibody and the commercially available CD19 immunoglobulin was studied.

RESULTS

The rabbit was first bled ten days after the first immunization. Both the baseline and immunized rabbit serum at 1:150 dilution showed high background when reacted with leucocytes extracted from whole blood of a normal individual. However, the immunized rabbit serum stained 12% of the cells in region 2 as compared to only 4% stained by the normal rabbit serum. This shows the presence of an antibody that is staining the lymphocyte population. The rabbit was bled and the titre was tested again 43 days after the first immunization. Each dilution of the immunized rabbit serum is paired with a

similar dilution of the baseline sample which acts as a control. Positive reaction was represented by the cells with fluorescence intensity above the cut-off point set using the baseline serum. The percentage of cells in each region which was positive when reacted with the immunized rabbit serum at different dilutions was illustrated in Table 2. At the 1:150 - 1:600 dilution of the immunized and baseline serum, a high background was observed i.e. the cells in all four regions reacted strongly with the immunized rabbit serum showing fluorescence intensity between 10^2 to 10^3 . This was higher than the fluorescence intensity (less than 10^2) of the cells reacted with the baseline serum. From dilutions above 1:4800, only cells in region one and two showed definitive reaction. The rest of the cells in region three and four had low fluorescence intensity i.e. unlabelled cells. The immunized rabbit serum had a very high titre i.e. more than 1:38400. The rabbit was given a booster and was sacrificed 52 days after the first immunization. We decided to use the 52 days serum at a dilution of 1:10000 to measure the percentage of B lymphocytes in 13 leukaemic patients diagnosed as different types of leukaemia by immunophenotyping the cell surface markers using commercial monoclonal antibody (Becton Dickinson, USA).

The immunized rabbit serum was found not to be reactive with T lymphocytes as shown in Fig. 2 but with cells extracted from a B-Chronic

TABLE 2: Percentage of cells in each region labelled by the immunized rabbit serum

Dilution	Percentage of positive reacting cells			
	Region 1	Region 2	Region 3	Region 4
1:150	99	99	95	68
1:600	97	99	88	45
1:4800	12	10	0.6	0.2
1:19200	10	4	1.4	0.3
1:38400	7.2	4.9	1.2	0.4

Lymphocytic Leukaemia (Fig. 3). We tested the performance of the immunized rabbit serum by comparing the percentage of B lymphocytes labelled by the commercial monoclonal anti-CD19 and the in-house polyclonal antibody (Table 3). There was a significant positive correlation between the proportion of B cells labelled with anti-CD19 and the immunized rabbit serum (n=13, r=0.7, p=0.02). The intensity of labelling between the anti-CD19 and the immunized rabbit serum as compared with their control are represented in Figs. 4 and 5 respectively.

We noted that the baseline serum had a higher percentage of non-specific staining (fluorescence

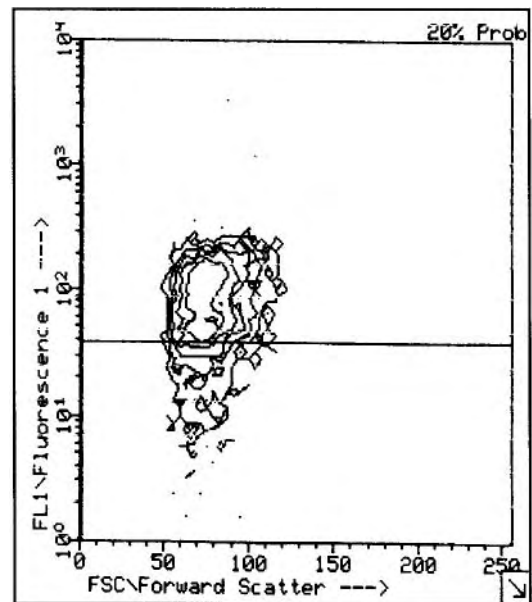


FIG. 3: Immunolabelling of cells extracted from a B-CLL patient with the in-house polyclonal antibody.

intensity range: 1.5-10 units, mean fluorescence intensity peak: 3.1 units) when compared to the commercial monoclonal control (IgG_{2a}) (range: 1.0-2.0 units, mean: 1.5 units). We find that the intensity range for the commercial anti-CD19

TABLE 3: Comparison between the percentage of lymphoid cells from 13 leukaemic patients reacting positively with commercial anti-CD19 and with in-house antibody diluted at 1:10000

Cases	CD19#	IRS*	Cases	CD19	IRS
1	65	38	8	78	67
2	90	18	9	94	67
3	94	40	10	68	73
4	87	21	11	0	5
5	90	70	12	7	6
6	77	76	13	2	14
7	88	61			

Case 1-7 : Acute Lymphoblastic Leukaemia of B lineage

Case 8-9 : B-Chronic Lymphocytic Leukaemia

Case 10 : Chronic Myeloid Leukaemia with Acute Lymphoblastic Leukaemia Transformation (CD19 & CD10 positive)

Case 11 : Chronic Myeloid Leukaemia with Acute Lymphoblastic Leukaemia Transformation (CD10 positive)

Case 12 : T-suppressor (CD8) Acute Lymphoblastic Leukaemia

Case 13 : Acute Myeloid Leukaemia

CD19# = Commercial monoclonal antibody against CD19 antigen/pan B (Becton Dickinson, USA)

IRS* = Immunized rabbit serum (immunogen: purified B lymphocytes)

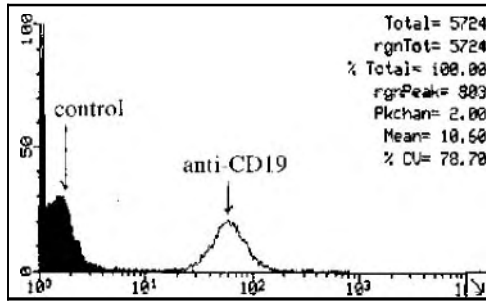


FIG. 4: Fluorescence intensity of the commercial CD19 and its control (IgG_{2a}).

was between 2-250 fluorescence units (mean fluorescence intensity peak = X8X units) and for the in-house polyclonal was between 6-30 fluorescence units (mean fluorescence intensity peak = 12.4 units). Fluorescence units are relative although with proper calibration, the fluorescence units can be directly related to the number of fluorochromes on each cell which is the amount of antibody labelling each cell.¹⁷

DISCUSSION

The outcome of any immunization may be ascribed to variations in individual animal response, immunogen, the choice of animal, the route of injection, the adjuvant and the dosage schedule.^{15,18} Rabbit was chosen because it was easily available, cheap, easy to care, robust in the face of quite intensive immunizations and easy to bleed.¹⁷ Particulate or cellular antigens are usually intensely immunogenic with the ability to invoke a rapid response. Intravenous immunization is believed to produce the most efficient immune response.¹⁵ Our study showed the presence of an antibody ten days after the first immunization that labelled 12% of the cells in region 2 as compared to only 4% labelled by non-immunized rabbit serum. A working antiserum was obtained in about two months.

An antibody needs to be characterized by determining its specificity and titre. The specificity needs to be established in order to evaluate the usefulness of the antibody.¹⁹ In our study, the specificity of the antibody was tested by studying the amount of positive reaction in different types of leucocytes from a normal individual and with purified T and B lymphoid cells. It was shown that at higher dilutions the majority of cells showing significant reaction

were lymphocytes. The antibody did not react with T lymphocytes, T Acute Lymphoblastic Leukaemia cells and myeloblasts but with B Chronic Lymphocytic Leukaemia cells and B Acute Lymphoblastic Leukaemia cells. This shows that the antibody was specific for B lymphocytes. It is interesting to note two cases of Chronic Myeloid Leukaemia with lymphoid blast transformation: one case showed CD10 and CD19 markers and the other showed only CD10 markers. The cells from the latter did not react with our antiserum. This is consistent with the immunogen we had used which were purified H lymphocytes from a B Chronic Lymphocytic Leukaemia patient that lack the CD10 antigens on their surfaces.

The titre provides information on how much material we have available and the degree of immune response of the immunized animal. The immunized rabbit had responded well to yield an antibody with high titre because positive reaction was still detected at dilutions of 1:38400. Suitable dilution for the working antiserum was found to be between 1:4800 and 1:38400. The rabbit serum was stored at -20°C and we did not experience any significant loss activity over a period of 19 months.

The significant correlation between the percentage of B lymphocytes labelled by the commercial anti-CD19 monoclonal antibody and the in-house polyclonal anti-B showed that the performance of the in-house polyclonal was satisfactory. However, we found that the percentage of B cells labelled by the in-house anti-B polyclonal antibody was less than the percentage labelled by the commercial anti-CD19. The mean fluorescence intensity of the commercial antibody was higher than that of the in-house polyclonal antibody. This observation is contradictory to the principle of immunostaining

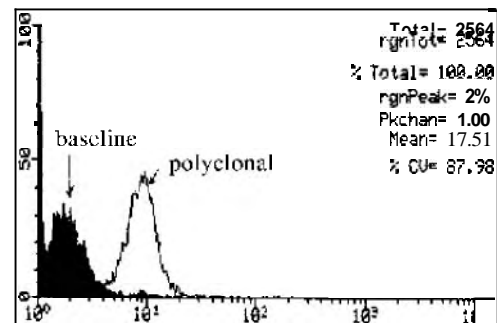


FIG. 5: Fluorescence intensity of the in-house polyclonal antibody and its control (baseline)

where indirect labelling allows the attachment of several times more fluorochromes to each antigen on the cell surface. This causes the amplification of the fluorescence intensity.¹⁶ Conventional antiserum will not only have antibodies to several determinants but also a family of antibodies of different structure and avidity which compete for each individual determinant.²⁰ Therefore the lower specificity and avidity of the antiserum may contribute to the lower fluorescence intensity although the method chosen was a more sensitive one as compared to the direct labelling method of the commercial monoclonal antibody. Some subsets of lymphocytes (notably natural killer cells) have receptors capable of binding immunoglobulins via their Fc receptors. This is especially a problem when using rabbit polyclonal antibodies; less with goat immunoglobulins and even less with monoclonal antibodies.¹⁶ This may attribute to the higher non-specific binding as seen in baseline and the polyclonal sera.

ACKNOWLEDGEMENTS

This research project was fully supported by the IRPA grant No. 3-07-03-059 from the Ministry of Science, Technology and Environment, Malaysia. We wish to express our gratitude to the staff of the Animal House, UKM for their help in this project.

REFERENCES

1. Keren DF, ed. *Flow Cytometry in Clinical Diagnosis*. 1st ed. Chicago: American Society of Clinical Pathologists, 1989.
2. Hoffman RA, Kung FC, Hansen WP, *et al*. Simple and rapid measurement of human T-lymphocytes and their subclasses in peripheral blood. *Proc Natl Acad Sci USA* 1980; 77(8): 4914-7.
3. Loken MR, Stall AM. Flow cytometry as an analytical and preparative tool in immunology. *J Immunol Methods* 1982; 50: R85-112.
4. Lovett EJ, Schnitzer, Keren DF, *et al*. Application of flow cytometry to diagnostic pathology. *Lab Invest* 1984; 50: 115-40.
5. Parks DR, Herzenberg LA. Fluorescence activated cell sorting: Theory, experimental optimization and applications in lymphoid cell biology. *Methods Enzymol* 1984; 109: 197-241.
6. Lim VC, Cheong SK. Analysis of human white blood cells by flow cytometry. Symposium on trends in biotechnology in the Asia-Pacific Region, Serdang. 1990: 73 (Abstract).
7. Cheong SK, Lim YC, Hamidah NH, Ainoon O. Flow cytometry in the immunodiagnosis of acute leukaemias. In: Edariah AB *et al*, ed. *Proceedings of the Second Medical Colloquium*, Kuala Lumpur, 1990: 139-40.
8. Cheong SK, Lim YC, Hamidah NH, Ainoon O. Immunophenotyping of acute lymphoblastic leukaemias by flow cytometry. Silver Jubilee Malaysia-Singapore Congress of Medicine, Kuala Lumpur, 1991: 164 (Abstract).
9. Kidd PG, Vogt RFJ. Report of the workshop on the evaluation of T cell subsets during HIV infection and AIDS. *Clin Immunol Immunopathol* 1989; 52: 3-9.
10. Melendro EI, Saldate C, Rivero SJ, Alarcon-Segovia D. T-cell subpopulations in the peripheral blood of patients with connective tissue disease as determined by flow cytometry using monoclonal antibodies. *Clin Immunol Immunopathol* 1983; 27: 340-7.
11. Anonymous. *Methods in Clinical Flow Cytometry*. San Jose: Becton Dickinson Immunocytometry Systems, 1988.
12. Hoffman RA, Kung PC, Hansen WP, Goldstein G. Simple and rapid measurement of T lymphocytes and their subclasses in peripheral blood. *Proc Natl Acad Sci* 1980; 77: 4914-7.
13. Hoffman KA, Hansen WP. Immunofluorescent analysis of blood cells by flow cytometry. *Int J Immunopharmacol* 1981; 3: 249-54.
14. Clark M, Waldman H. Production of monoclonal antibodies. In: Beverly CPL, ed. *Methods in Haematology: Monoclonal Antibodies*. Oxford: Churchill Livingstone, 1980: 1-20.
15. Hum RAL, Chantler SH. Production of reagent antibodies. *Methods Enzymol* 1980; 70: 104-42.
16. Ault KA. Application in immunology and lymphocyte analysis. In: Melamed MR, Lindmo T, Mendelsohn ML, eds. *Flow Cytometry and Sorting*. New York: Wiley-Liss, 1990: 685-96.
17. Loken MR. Cell surface antigen and characterization of leucocyte populations by flow cytometry. In: Beverly CPL, ed. *Methods in Haematology: Monoclonal Antibodies*. Oxford: Churchill Livingstone, 1986: 132-44.
18. Johnstone A, Thorpe R. *Immunochemistry in Practice*. Oxford: Blackwell Scientific Publications, 1982: 27-40.
19. Zola H, Brooks D. Techniques for production and characterization of monoclonal hybridoma antibodies. In: Hurrell JGR, ed. *Monoclonal Hybridoma Antibodies: Techniques and Applications*. Florida: CRC Press, Inc., 1982: 1-57.
20. Campbell AM. General properties and applications of monoclonal antibodies. In: Burdon RH, van Knippenberg PH, eds. *Laboratory techniques in biochemistry and molecular biology: Monoclonal antibody technology*. Amsterdam: Elsevier, 1984: 1-32.