Immunoperoxidase detection of T and B cells in blood compared with conventional methods

PK HUI*, JWM LAWTON†

*From the *Haematology and †Immunology Units, Department of Pathology, University of Hong Kong*

SUMMARY Peripheral blood T and B cells were enumerated in 26 normal adults by conventional immunological markers (erythrocyte rosette and surface membrane immunoglobulin) and by monoclonal markers (T11 and B1) using both membrane immunofluorescence of cells in suspension and immunoperoxidase staining of dried, fixed, cytocentrifuged cells after separation from blood by buoyant density centrifugation. The results of immunochemistry were determined independently from the results of erythrocyte rosetting and membrane immunofluorescence techniques. A high correlation was found between all three methods for enumerating T cells (erythrocyte rosetting, T11/immunoperoxidase, and T11/indirect immunofluorescence). Correlation was less good between the results of the three methods for enumerating B cells; surface membrane immunoglobulin and B1/indirect immunofluorescence techniques showed the highest correlation. The results suggested that B cells may be preferentially lost during the extensive cell washings entailed in the indirect immunofluorescence procedures. Immunochemical methods, using monoclonal antibodies, are useful for enumerating lymphocyte subsets in blood, particularly when permanent records are desired.

Conventionally, peripheral blood T and B lymphocytes are identified by the sheep erythrocyte rosetting technique and the demonstration of surface membrane bound immunoglobulin, respectively.¹ The recent development of monoclonal antibodies directed against various lymphocyte surface antigens has provided an additional and precise means of defining lymphocyte subsets.^{2,3} The typing of lymphocytes by monoclonal antibodies is usually based on the immunofluorescence technique, whereby the labelled cells are quantitated either by fluorescence microscopy or by a fluorescence activated cell sorter.^{4,5} These same lymphocyte surface markers may also be shown by immunochemical techniques using air dried cell preparations.⁶ The immunoperoxidase method is technically simpler than the immunofluorescence technique; in the latter viable cells must be labelled in suspension, which entails many time consuming cell washings. Moreover, additional steps have to be taken in the immunofluorescence method to identify other cell types such as monocytes. The immunoperoxidase method, in contrast, is performed on a relatively small number of fixed dead cells.

To explore the potential use of the immunoperox-

idase technique we have carried out a comparative study of the immunoperoxidase method and the conventional immunological methods in the enumeration of peripheral blood B and T cells. As part of this study we correlated results of immunocytochemistry and immunofluorescence in detecting markers defined by two monoclonal antibodies and compared the two methods in terms of practical convenience for routine clinical laboratory use.

Material and methods

BLOOD SAMPLES

Venous blood samples were obtained from 26 healthy adult volunteers. A 20 ml portion of each blood sample was anticoagulated with heparin (10 U/ml) and sent to the immunology laboratory for rosetting and immunofluorescence studies. A 3 ml portion of each sample was anticoagulated in edetic acid and sent to the haematology laboratory for immunoperoxidase studies. The two samples were processed and the results were assessed independently.

PREPARATION OF CELLS

Peripheral blood mononuclear cells from edetic acid and heparin anticoagulated blood were obtained by

buoyant density centrifugation over Ficoll-Hypaque (specific gravity 1.077).⁷ For immunoperoxidase staining the separated mononuclear cells were diluted to 10^5 cells per millilitre in normal saline, and then portions were centrifuged on to glass slides in a cytocentrifuge (Shandon-Elliott Cheshire, UK). For rosetting and immunofluorescence techniques, the harvested mononuclear cells were washed twice in Hank's balanced salt solution, adjusted to 4×10^6 cells per millilitre, and incubated with polystyrene latex at 37°C for 20 min.

ANTIBODIES

The two monoclonal reagents T11 and B1 were purchased from Coulter Corporation, Florida, USA. T11 has a specificity against the erythrocyte receptor protein⁸ and B1 has a major specificity directed against B lymphocytes.⁹ The second antibody, peroxidase conjugated goat antimouse IgG, was purchased from Tago, California, USA. Fluorescein isothiocyanate conjugated rabbit antihuman Ig(G + A + M) was purchased from Behringwerke AG, Marburg, West Germany. The second antibody for indirect immunofluorescence was fluorescein isothiocyanate conjugated rabbit antimouse IgG (Miles-Yeda Ltd, Rehovot, Israel).

ERYTHROCYTE ROSETTING TECHNIQUE

This was performed according to the method of Kaplan and Clark¹⁰ using amino-ethylthiuronium-bromide treated sheep erythrocytes. Sheep erythrocytes were not more than two weeks old when used. Mononuclear cells were incubated together with treated erythrocytes for 3h at 4°C and after resuspension the number of T cells (erythrocyte rosettes) was determined by counting a total of 200 cells in a wet preparation with toluidine blue to stain nucleated cells.

IMMUNOFLUORESCENCE TO DETECT SURFACE MEMBRANE IMMUNOGLOBULIN

B cell surface membrane immunoglobulin was detected by direct immunofluorescence¹¹ using fluorescein isothiocyanate conjugated rabbit antihuman Ig (G + A + M). Separated and washed blood mononuclear cells were incubated with polystyrene latex at 37°C for 20 min and then left at ambient temperature for 30 min; this procedure was to allow monocytes to ingest polystyrene (for later identification) and to allow adsorbed immunoglobulin to elute from the surface of lymphocytes. Cells were then spun down, the supernatant was removed, and the conjugate (diluted 1/8) was added directly to the cell pellet. The mixture was incubated on ice for 30 min and the cells washed three times in phosphate buffered saline (PBS) azide with 1% bovine

serum albumin and suspended in buffered glycerol, before reading membrane fluorescence under epifluorescence illumination. One hundred cells were counted.

IMMUNOFLUORESCENCE METHOD FOR T11 AND B1 MARKERS

The cell membrane epitopes defined by T11 and B1 monoclonal antibodies were detected by indirect immunofluorescence using a technique similar to that of direct immunofluorescence. After incubation with polystyrene latex the mononuclear cells were adjusted to 10×10^6 /ml. The monoclonal antibody was added to 100 μ l of the cell suspension to give a final dilution of 1/50. After incubation at 4°C for 30 min the cells were washed twice and the second antibody (fluorescein isothiocyanate labelled antimouse immunoglobulin), diluted 1/20, was added to the cell pellet. After further incubation at 4°C for 30 min the cells were washed three times and suspended in FA mounting fluid for fluorescence microscopy.

In the erythrocyte rosette and immunofluorescence techniques, monocytes were identified by their morphology, the presence of ingested polystyrene particles, and the irregular, granular, non-specific surface membrane fluorescence.

IMMUNOPEROXIDASE METHOD FOR T11 AND B1 MARKERS

Air dried cytocentrifuged preparations of mononuclear cells were fixed for 30 s in pH6.6 buffered formalin-acetone at 4°C,^{6,12} after which the cells were stained for T11 and B1 by an indirect immunoperoxidase technique as described previously.⁶ In brief, smears were first incubated for 1 h with 100 μ l of monoclonal antibody diluted 1/10 in PBS at pH 7.2. The smears were then washed in PBS for 5 min and incubated for 45 min with 100 μ l of peroxidase conjugated goat antimouse IgG diluted 1/15 in PBS. The smears were washed again for 5 min in PBS. The peroxidase conjugate was stained by a substrate solution containing 3-amino-9-ethylcarbazole (Sigma) and hydrogen peroxide.¹³ Stained smears were counterstained in Mayer's haematoxylin and mounted in glycerin jelly. Slides were examined under oil immersion at $\times 1000$ magnification using a Leitz Laborlux microscope. Positively stained cells showed a reddish brown precipitate on the cell surface with the marginal area of the cell more intensely stained. Two hundred cells were examined and the percentage of positively stained cells was determined. Monocytes were excluded from the count by morphological criteria.

TITRATION OF ANTIBODIES

The working dilutions of antibodies used in both the immunofluorescence and immunoperoxidase methods were determined by titrating the individual reagents in doubling dilutions. The working dilution was the highest dilution on the "plateau" of high positivity.

STATISTICAL ANALYSIS

Results are expressed as mean and standard deviation. Differences in means were compared using Student's *t* test for paired observations. Correlation between results obtained by different methods was expressed as Pearson's coefficient of correlation. The significance of the correlation was estimated by the *t* test. Linear regression was calculated by the method of least squares.

Results

Tables 1 and 2 show the results of the three methods in enumerating peripheral T and B cells. The percentage of T11 positive cells by the indirect immunofluorescence method was significantly higher than that obtained by the indirect

immunoperoxidase method ($p < 0.01$) and by the erythrocyte rosetting technique ($p < 0.01$). No significant difference was found between the percentage of erythrocyte receptor positive cells and the T11 positive cells by the immunoperoxidase method. The percentage of B1 positive cells by the immunoperoxidase method was significantly higher than the percentage of surface membrane immunoglobulin positive cells ($p < 0.01$) and B1 positive cells by the immunofluorescence technique ($p < 0.01$). The percentage of B1 positive cells by the immunofluorescence technique was significantly lower than that of surface membrane immunoglobulin positive cells ($p < 0.01$).

Table 3 shows the correlation between the results obtained by the various methods. Despite the discrepancies observed in the means, the various methods used in quantitating T and B lymphocytes showed significant positive linear correlation with their relevant counterparts. Higher correlation coefficients ($r > 0.99$) were found between all three methods for enumerating T cells. Of the three methods for enumerating B cells, the surface membrane immunoglobulin and B1 (immunofluorescence) techniques showed the highest correlation ($r = 0.977$).

Table 1 Enumeration of peripheral blood T lymphocytes by three different methods

	Erythrocyte rosetting	T11 (immunoperoxidase)	T11 (immunofluorescence)
% positive (n = 26)	72.8	71.4	79.7
Range	56-83	56-84	55-93
SD	8.84	7.87	9.66
Significance*	—	NS	$p < 0.01$

NS = not significant.

*Significance in comparison with erythrocyte rosette method.

Table 2 Enumeration of peripheral blood B lymphocytes by three different methods

	Surface membrane immunoglobulin	B1 (immunoperoxidase)	B1 (immunofluorescence)
% positive (n = 26)	14.7	17.3	12.6
Range	6-35	10-30	6-29
SD	7.24	4.72	6.96
Significance*	—	$p < 0.01$	$p < 0.01$

*Significance in comparison with surface membrane immunoglobulin.

Table 3 Correlation of results of the methods used to enumerate peripheral blood T and B lymphocytes (n = 26)

Correlation between (y/x)	r	Linear regression	p
ER/T11 (IF)	0.994	$y = 0.908x + 0.35$	$0.01 > p > 0.001$
ER/T11 (IP)	0.994	$y = 1.018 + 0.083$	$0.01 > p > 0.001$
T11 (IF)/T11 (IP)	0.993	$y = 1.11x + 0.106$	$0.01 > p > 0.001$
smIg/B1 (IF)	0.997	$y = 1.11x + 0.701$	$0.01 > p > 0.001$
smIg/B1 (IP)	0.886	$y = 0.832x + 0.274$	$0.01 > p > 0.001$
B1 (IF)/B1 (IP)	0.878	$y = 0.706x + 0.326$	$0.01 > p > 0.001$

IP = indirect immunoperoxidase.

IF = indirect immunofluorescence.

ER = erythrocyte rosette.

smIg = surface membrane immunoglobulin.

r = Pearson's coefficient of correlation.

Discussion

Immunoperoxidase staining to detect lymphocyte surface markers offers several advantages over the conventional immunological methods. Firstly, the immunoreactivity of the various cell surface antigens in cell smears is fairly stable on storage at room temperature^{6 14 15} and stable for long periods if slides are stored frozen¹⁶; staining may therefore be performed in batches with resultant savings of staff time. Secondly, cell morphology and surface markers may be studied simultaneously so that irrelevant cell types may be excluded easily by morphological criteria and stained smears may be filed as a permanent record. Thirdly, a smaller quantity of blood is required to prepare smears for staining by a panel of antibodies; this is particularly advantageous when studying paediatric samples. Furthermore, the immunoperoxidase technique does not require time consuming washings of living cells and the reading of the final preparation is less prone to subjective error than the reading of immunofluorescence.

The results of our study confirm the wide variation in proportions of T and B cells in the blood of normal subjects,¹⁷ and the ranges of values which we obtained using the immunoperoxidase technique were comparable to those obtained by Erber *et al*¹⁶ using the immunoalkaline phosphatase technique on whole blood smears.

Correlation was good between the results of the various methods to detect T cell markers ($r > 0.99$), which was to be expected since T11 has specificity against the erythrocyte receptor of T cells.⁷ Correlation between the methods to detect B cell markers was less satisfactory. B1 is not directed against surface membrane immunoglobulin; however, this fact alone does not account for the discrepancy observed and the explanation may be a technical one. The immunofluorescence method entails more washing of cells, which could lead to a preferential loss of B cells during washing and decanting because of their lower density.¹⁸ In addition, B cells may be preferentially lost during incubation and washing procedures because of their well known tendency to adhere to glass or plastic surfaces.^{19 20 21} Table 4 shows that the

indirect immunofluorescence method, entailing eight cell washings, gave the lowest B cell count, the highest T cell count, and therefore the lowest B:T ratio. The surface membrane immunoglobulin method, which entailed five washings, gave a mean B cell count significantly higher than B1 (immunofluorescence method) but lower than B1 (immunoperoxidase method), which required no cell washing. The higher B cell counts given by B1 (immunoperoxidase method) may also be due to the fact that antigen deep in the cell membrane or in the cytoplasm is detected by the cell smear method but not by membrane immunofluorescence.

The results of this study clearly show that immunochemical methods give normal values significantly different from conventional immunological techniques in the enumeration of lymphocyte subsets, and these differences may be explained by technical factors. Notwithstanding this fact, in our hands the immunoperoxidase method has proved to be a useful additional way of enumerating lymphocyte subsets. It is of particular value when studying small blood samples and the immunocytochemical technique may well prove to be the method of choice in typing lymphocytic leukaemias where a permanent record is desired.

References

¹ Brown G, Greaves MF. Enumeration of absolute numbers of T and B lymphocytes in human blood. *Scand J Immunol* 1974;3:161-7.
² Diamond BA, Yelton DE, Scharff MD. Monoclonal antibodies: A new technology for producing serologic reagents. *N Engl J Med* 1981;304:1344-9.
³ Aisenberg AC. Cell-surface markers in lymphoproliferative disease. *Current Concepts in Immunology* 1981;304:331-6.
⁴ Van Agthoven A, Terhorst C, Reinherz EL, Schlossman SF. Characterisation of T cell surface glycoproteins T1 and T3 present on all human peripheral T lymphocytes and functional mature lymphocytes. *Eur J Immunol* 1981;11:18-21.
⁵ Reinherz EL, Kung TC, Goldstein G, Levey RH, Schlossman SF. Discrete stages of human interthymic differentiation: Analysis of normal lymphocytes and leukaemic lymphoblasts of T lineage. *Proc Natl Acad Sci USA* 1980;77:1588-92.
⁶ Hui PK, Joyce R. A simple immunocytochemical staining procedure for lymphoid cell surface markers done on cell smears. *J Clin Pathol* 1984;37:708-9.

Table 4 Number of cell washings and the B:T cell ratio

	Immunoperoxidase method	Surface membrane immunoglobulin (direct immunofluorescence method)	Immunofluorescence monoclonal (indirect)
No of centrifugations	0	5	8
% B cells	17.3	14.7	12.6
% T cells	71.4	NA	79.7
B:T	0.242	0.188*	0.159

*Surface membrane immunoglobulin positive/erythrocyte rosette positive cells.

- ⁷ Boyum A. Separation of white blood cells. *Nature* 1964;**204**:793-4.
- ⁸ Howard FD, Ledbetter JA, Wong J, Bieber CP, Stitson ED, Hertzberg LA. A human T lymphocyte differentiation marker defined by monoclonal antibodies that block rosette formation. *J Immunol* 1981;**126**:2117-21.
- ⁹ Stashenko P, Nadler LM, Hardy R, Schlossman SF. Characterisation of a human B lymphocyte specific antigen. *J Immunol* 1980;**125**:1678-85.
- ¹⁰ Kaplan ME, Clark C. An improved rosetting assay for detection of human T lymphocytes. *J Immunol Methods* 1974;**5**:131-5.
- ¹¹ Peacock JE, Tomar RH. *Manual of Laboratory Immunology*, Philadelphia: Lea and Febiger, 1980.
- ¹² Yam LT, Li CY, Crosby WH. Cytochemical identification of monocytes and granulocytes. *Am J Clin Pathol* 1971;**55**:283-90.
- ¹³ Graham RC, Lundholm U, Karnovsky MJ. Cytochemical demonstration of peroxidase activity with 3-amino-9-ethylcarbazole. *J Histochem Cytochem* 1965;**13**:150-2.
- ¹⁴ Li CY, Ziesmer SC, Yam LT, English MC, Janckila AJ. Practical immunocytochemical identification of human blood cells. *Am J Clin Pathol* 1984;**81**:204-12.
- ¹⁵ Moir DJ, Ghosh AK, Abdulaziz Z, Knight PM, Mason DY. Immunoenzymatic staining of haematological samples with monoclonal antibodies. *Br J Haematol* 1983;**55**:395-410.
- ¹⁶ Erber WN, Pinching AJ, Mason DY. Immunocytochemical detection of T and B cell populations in routine blood smears. *Lancet* 1984;i:1042-5.
- ¹⁷ Bongers V, Bertrams J. The influence of common variables on T cell subsets by monoclonal antibodies. *J Immunol Methods* 1984;**67**:2443-53.
- ¹⁸ Yu D, Peter JB, Paulus HE, Nies KM. Human lymphocyte subpopulations. Study of T and B cells and their density distribution. *Clin Immunol Immunopathol* 1974;**2**:333-6.
- ¹⁹ Greaves MF, Brown G. Separation of human B and T lymphocytes. *J Immunol* 1984;**112**:420-6.
- ²⁰ Eisen SA, Wender HJ, Parker CW. Isolation of pure human peripheral blood T lymphocytes using nylon wool columns. *Immunology Communications* 1972;**1**: 571-5.
- ²¹ Iwantani Y, Amino N, Mori H, Asari S, Ina K, Ennyu K, Miyai K. Effects of various isolation methods for human peripheral lymphocytes on T cell subsets determined in a fluorescence activated cell sorter, and demonstration of a sex difference of suppressor/cytotoxic T cell. *J Immunol Methods* 1982;**54**:31-42.

Requests for reprints to: JWM Lawton, Department of Pathology, University of Hong Kong, Hong Kong.