Detection of surface immunoglobulins of human lymphoid cells: a comparative study of live and fixed cells using a direct immunoperoxidase procedure

GUY LAURENT, MARIE FRANÇOISE GOURDIN,* FELIX REYES*

From the Service d'Hématologie Clinique, CHU Purpan, Toulouse, and the *INSERM U91, CHU Henri Mondor, Creteil, France

SUMMARY Surface immunoglobulins (Ig) of normal and malignant lymphoid cells were detected on prefixed, smeared (method A) and live (method B) cell suspensions; the results were compared with regard to staining patterns, specificity and sensitivity. In both methods surface Ig were detected by a direct immunoperoxidase procedure using conjugated purified antibody. Although method A has practical advantages, method B is more sensitive. The reasons for this discrepancy are discussed in relation to surface Ig denaturation and redistribution.

The demonstration of surface immunoglobulin (Ig) is essential in identifying B lymphocytes and classifying lymphoid malignancies. For that purpose surface immunofluorescence (IF) of live cells has been widely used.¹

On the other hand immunoperoxidase procedures (IPX) have been developed and proposed as an alternative to IF because of the following advantages: (i) storage of labelled material; (ii) feasibility with standard equipment of optical microscopy; (iii) simultaneous morphological identification based on routine counterstaining. Thus, IPX procedures have spread dramatically in haematopathology laboratories, major applications including the demonstration of cytoplasmic antigens in fixed, embedded tissue sections² such as immunoglobulins of lymphoma cells.³ In contrast surface Ig have been less frequently studied by IPX either on prefixed⁴⁻⁷ or live cell suspensions,^{8,9} although a comparative study⁸ found IPX and IF to be comparable in demonstrating surface Ig of live cells.

Finally it appears from published reports that in most cases IF is employed for staining live cells and IPX for fixed cells.

In a recent study using the IPX staining of fixed cells,⁶ the finding of a surprisingly low proportion of surface Ig-positive cases among CLL samples prompted us to compare the results obtained by staining live and prefixed cells of identical samples.

Material and methods

SAMPLES (TABLE 1)

Daudi cells (human B cell line established from Burkitt's lymphoma) were maintained in RPMI 1640 with 10% fetal calf serum. Buffy coat mononuclear cell suspensions were obtained from three normal individuals and from patients with chronic

Table 1 Samples selected for staining of surface immunoglobulins

Samples	Conjugate (0.2 mg/ml)	% Stained cells	
		Method A	Method B
1 CLL	μ	61	85
2 "	μ	52	88
3 "	μ	43	73
4 "	μ	38	89
5 "	μ	5.5	80*
6 "	μ	0	60*
7 "	μ	0	90*
8 "	μ	0	36
9 "	μ	0	66
10 "	μ†	20	40
11 HCL	γ	30	82
12 "	γ	80	94
13 "	γ	76	72
14 "	γ	78	82
15 Lymphoma	μ	78	82
16 "	μ	52	88
17 Normal blood	μ	8	12
18 "	μ	5.5	14
19 "	μ	4.5	12
20 Daudi cells	μ	100	100

*Very faint staining.

†0.05 mg/ml.

lymphocytic leukaemia (CLL) (10 cases), nodular lymphoma with blood dissemination (2 cases) and hairy cell leukaemia (HCL) (4 cases). These patient samples were surface Ig-positive, with a polyclonal phenotype in HCL, and a monoclonal one in CLL and lymphoma cases as shown by the monotypic light chain-staining of smeared cells.⁷ Cells were washed three times and incubated for 30 min at 37°C in Hank's solution to remove cytophilic Ig.

ANTI-IMMUNOGLOBULIN ANTIBODIES

Monospecific anti-IgM and anti-IgG antibodies were affinity-purified from rabbit hyperimmune sera as already described by solid immunoabsorption.¹⁰ They were checked by immunodiffusion and by staining samples with a known monoclonal phenotype.⁷ Normal non-immune rabbit IgG (Sigma Chemical Co, St Louis, Missouri) were used in controls.

CONJUGATES

Rabbit anti- μ , anti- γ and normal IgG were conjugated to horseradish peroxidase (RZ3 Sigma Laboratory) using a two-step procedure.¹¹

IMMUNOSTAINING PROCEDURES

Method A Surface Ig were stained on fixed leucocyte smears, according to a modification of a technique previously described by Mason *et al.*⁴ Briefly, mononuclear cells were resuspended in Hank's solution in the presence of bovine serum albumin (BSA) (2.5%), smeared on glass slides and air-dried; alternatively smears could be stored at -80°C until required and then thawed for 10 min at room temperature before fixation. Smeared cells were fixed by a mixture of formol-acetone for thirty seconds at room temperature, washed in phosphate-buffered saline pH 7.4, then incubated for 30 min in methanol-H₂O₂ (0.3%) to block any endogenous peroxidase activity; they were then rinsed and air-dried. Afterwards, slides were overlaid by conjugate (100 μ l) for 30 min at room temperature, then rinsed in saline. The cytochemical detection of conjugated peroxidase was performed by laying the following medium on smears for 5 min in the dark: diaminobenzidine 10 mg in TRIS-HCl buffer, 0.05 M, 10 ml, 0.1 ml 1% H₂O₂, pH 7.6.¹²

Method B The detection of surface Ig was performed on live cell suspensions, according to Knowles *et al.*⁸ Mononuclear cells (5×10^6) suspended in Hank's solution were incubated with conjugate for 30 min at room temperature. Cells were centrifuged, washed three times in Hank's, resuspended in Hank's BSA (2.5%), smeared and air-dried. Subsequent steps of fixation, inhibition of

endogenous peroxidases and cytochemical detection were as in method A.

Method C The detection of surface Ig of live cells was performed with a parallel inhibition of antibody-induced surface Ig redistribution. For this purpose cells were incubated with conjugate in the presence of sodium azide (1%) at 4°C for 30 min,¹³ and further processed as in method B.

CONJUGATE DILUTIONS

Anti-immunoglobulin conjugates were tested over a range of dilutions; working optimal dilution was 0.2 mg/ml (1/5) corresponding to a dilution "plateau" over which a constant percentage of stained cells was found. In some experiments both methods (A and B) were assessed over a range of successive dilutions (Tables 2-6).

MICROSCOPIC EXAMINATION

Slides were counterstained by haematoxylin, coverslipped in DPX and examined by conventional light microscopy. At least 400 cells were counted in each experiment.

CONTROLS

Controls were provided by conjugated normal rabbit IgG used at 0.2 mg/ml.

Table 2 Amount and pattern of staining in Daudi cells (sample 20)

Conjugate concentration (anti- μ)	% Stained cells		
	Method A*	Method B*	Method C*
1/5	100 (diffuse)	100 (diffuse)	100 (diffuse)
1/20	100 (diffuse)	95 (diffuse)	100 (diffuse)
1/100	100 (diffuse)	100 (diffuse)	92 (diffuse)
1/200	30 (ring)	100 (caps)	27 (ring)
1/1000	0	50 (caps)	0
1/2000	0	0	0
1/10 000	0	0	0
Control			
Normal IgG	0	0.4	
1/5			

*Predominant staining pattern (see text).

Table 3 Amount and pattern of staining in CLL cells (sample 10)

Conjugate concentration (anti- μ)	% Stained cells		
	Method A	Method B	Method C
1/5	NT	NT	NT
1/20	20 (ring)	40 (cap)	26 (ring)
1/100	0	14 (cap)	6 (ring-cap)
1/200	0	3 (cap)	2 (cap)
1/1000	0	0	0
Control			
1/5	0	0.5	

NT = not tested.

Table 4 Amount and pattern of staining in HCL cells (sample 11)

Conjugate concentration (anti- γ)	% Stained cells		
	Method A	Method B	Method C
1/5	30 (ring)	82 (cap)	40 (ring-cap)
1/20	6 (ring)	61 (cap)	10 (ring-cap)
1/100	0	3 (cap)	2 (cap)
1/1000	0	0	0
Control			
1/5	0	9	

Table 5 Amount and pattern of staining in HCL cells (sample 12)

Conjugate concentration (anti- γ)	% Stained cells	
	Method A	Method B
1/5	80 (ring)	94 (cap-patches)
1/10	45 (ring)	84 (cap-patches)
1/20	30 (ring)	78 (cap)
1/100	5 (ring)	72 (cap)
Control		
1/5	0	6

Table 6 Amount and pattern of staining in CLL cells (sample 1)

Conjugate concentration (anti- μ)	% Stained cells	
	Method A	Method B
1/5	61 (ring)	85 (cap-patches)
1/20	10 (ring)	20 (cap-patches)
1/100	0	9 (cap)
1/1000	0	0
Control		
1/5	0	1

Results

STAINING PATTERNS

Method A Two morphological patterns of surface Ig staining were observed. The first consisted of a distinctive membrane-labelling: the cell periphery was clearly delineated by a dark brown margin contrasting with a pale cytoplasm. Such a ring pattern of staining was either strong (HCL, lymphoma, normal blood samples) or faint (CLL). The second pattern was represented by a diffuse staining with an abolition of the nuclear counter-staining as in Daudi cells and in some HCL cells. It is to be noted that four CLL samples out of 10 gave negative results.

Method B Cells displayed a distinctive staining corresponding to the antibody-induced redistribution of surface Ig: cells were typically stained by "caps" or had intermediate patterns with numerous patches

concentrating towards one pole of the cell. Typical "caps" were more frequently observed in Daudi and HCL cells than in CLL.

Method C Prevention of surface Ig redistribution in the presence of cold azide was performed in three samples: Daudi, CLL and HCL (Table 2-4). The great majority of positive cells indeed displayed a ring linear pattern of surface staining. However in each sample, the inhibition of surface Ig redistribution was incomplete and typical capped cells or intermediate forms were also seen.

SENSITIVITY

In every sample the percentage of surface Ig-positive cells was lower with method A than with method B (Table I). This striking difference was also found over the range of dilutions tested in several samples (see Tables 2-5). Furthermore results obtained with method C in three samples were found similar to those obtained with method A (Tables 2-4).

SPECIFICITY

Specificity was assessed by staining with conjugated normal IgG. In method A, no cell was stained whatever the sample under study. In methods B and C, less than 1% of Daudi and CLL cells were stained. However, the percentage of stained cells was slightly higher (6, 9%) in HCL samples, a result in accordance with the presence of Fc receptors on these cells.¹⁴

Discussion

During the last 10 years, IF staining of live cell suspensions has been employed as a routine method for the demonstration of surface Ig of human B cells.¹ Immunoperoxidase staining has been less frequently used for that purpose, although it appeared to have a similar sensitivity when performed on live cells.⁸ Actually IPX methods of staining have been extensively used in the study of fixed tissue sections and subsequently this approach has been extended to the study of fixed smears of cell suspensions in clinical haematology. In this context too, IPX was felt to offer similar advantages—that is, visualisation of cell morphology, permanence of staining, applicability to stored smears. It should be added to this that prefixation of cells may prevent Fc-mediated binding of conjugates¹⁵ therefore requiring not the use of F(ab)² antibody. Variants of IPX procedures have included the three-layer peroxidase-antiperoxidase method,^{5,6} the two-step¹⁶ and the one-step method.⁷

We were therefore interested in comparing on identical samples the results of surface Ig IPX-

staining performed on live (as in routine IF) and prefixed smeared cell suspensions.

This study confirmed the greater sensitivity of the immunostaining of live cells since: (i) the proportion of positive cells was lower in all CLL samples but one and normal individuals when stained by optimal conjugate concentration in method A; (ii) this was confirmed when tested over a range of dilutions. In addition, we confirmed that the staining pattern of prefixed cells did not always allow a clear distinction between surface and cytoplasmic staining in cells with large amounts of surface Ig such as Daudi cells;⁷ in such cases exposure of live cells to the conjugate before smearing caused the diffuse staining to be replaced by localised clumps thus identifying surface Ig.⁶

The IPX technique may lack sensitivity in demonstrating surface Ig when performed on smeared prefixed cells because of fixation-induced alteration of cells. It is known that aldehyde fixatives may partially destroy surface Ig in immunostaining of tissue sections^{17 18} and B cells with low amounts of surface Ig, such as CLL cells,¹⁹ may express faint or no surface staining.⁶ In keeping with this it has already been reported that surface IF of smeared cells is usually negative but can be positive in cells with large amounts of membrane Ig.⁵ However, it is known that the "patchy" pattern of surface Ig staining as shown by routine IF (live cells) results from a redistribution process induced by anti-Ig divalent antibody.²⁰ This process is seen by immunoelectron microscopy as a concentration of surface (Ig-anti-Ig) complexes at limited segments of the cell surface with invagination of the latter leading to the internalisation of the stained membrane.²¹ This continuing process leads gradually to a polar concentration of staining (capping). It may be that the patterns observed on live cells (patches, caps, and intermediate forms) are a result of a progressive concentration of Ig in some areas. CLL cells with a low amount of surface Ig (undetected by immunostaining of prefixed smears) would then become positively stained. The results that we obtained by method C are in agreement with this view: sodium azide and low temperature are able partially to inhibit the process of membrane movements; in these experimental conditions we observed an increased number of "ring" pattern, staining resembling that found in smeared prefixed cells. Moreover the proportions of surface Ig positive cells was lower than in method B (in which there was no inhibition of the redistribution process) and tended to be similar to that observed in method A (prefixed cells) when tested over the range of conjugate dilutions.

In conclusion prefixation of cells is likely to lower

the sensitivity of any method of surface Ig staining as a result of antigenic alteration and abolition of cell membrane movements. Although the IPX technique applied to prefixed smeared cells offers a number of advantages—applicability to stored samples, visualisation of cell morphology, simultaneous study of cytoplasmic antigens, loss of surface Fc receptors—it should not be recommended as the sole routine method for screening cells from immunoproliferations that may have variable amounts of surface Ig.

We acknowledge the secretarial assistance of M Segear. This study was supported by a grant (900 651 011) from Université Paris Val de Marne.

References

- 1 Preud'Homme JL, Seligmann M. Surface immunoglobulins on human lymphoid cells. *Prog Clin Immunol* 1974;2:121-74.
- 2 De Lellis RA, Sternberger LA, Mann RB, Banks PM, Nakane PK. Immunoperoxidase techniques in diagnostic pathology. Report of a workshop sponsored by the National Cancer Institute. *Am J Clin Pathol* 1979;71:483-8.
- 3 Taylor CR. An immunohistological study of follicular lymphoma, reticulum cell sarcoma and Hodgkin's disease. *Eur J Cancer* 1976;12:61-75.
- 4 Mason DY, Farrell C, Taylor CR. The detection of intracellular antigens in human leucocytes by immunoperoxidase staining. *Br J Haematol* 1975;31:361-70.
- 5 Mason DY, Labaume S, Preud'Homme JL. The detection of membrane and cytoplasmic immunoglobulins in human leukocytes by immunoperoxidase staining. *Clin Exp Immunol* 1977;29:413-21.
- 6 Mason DY, Leonard RCF, Laurent G, Gourdin MF. Immunoperoxidase staining of surface and intracellular immunoglobulin in human neoplastic lymphoid cells. *J Clin Pathol* 1980;33:609-16.
- 7 Laurent G, Gourdin MF, Reyes F. Immunoperoxidase detection of immunoglobulins in cells of immunoproliferative diseases. A comparison between conjugate and nonconjugate (PAP) procedures. *Am J Pathol* 1980;74:265-74.
- 8 Knowles DM, Winchester RJ, Kunkel MG. A comparison of peroxidase and fluorochrome conjugated antisera for the demonstration of surface and intracellular antigens. *Clin Immunol Immunopathol* 1977;7:410-25.
- 9 Middleditch PR, Minard BJ, Cawley LP, Bastian FO. Horseradish peroxidase labelled antisera: a diagnostic method for evaluating the percentages of B lymphocytes in peripheral blood. *J Histochem Cytochem* 1979;27:689-90.
- 10 Reyes F, Lejonc JL, Gourdin MF, Mannoni P, Dreyfus B. The surface morphology of human B lymphocyte as revealed by immunoelectron microscopy. *J Exp Med* 1975;141:392-410.
- 11 Avrameas S, Ternynck T. Peroxidase-labelled antibody and Fab conjugates with enhanced intracellular penetration. *Immunochemistry* 1971;8:1175-9.
- 12 Graham RC, Karnovsky MJ. The early stages of absorption of injected HRP in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J Histochem Cytochem* 1966;14:429-35.

- ¹³ Preud'Homme JL, Seligmann M. Immunoglobulins on the surface of lymphoid cells in Waldenström's macroglobulinemia. *J Clin Invest* 1972;**51**:701-5.
- ¹⁴ Jaffe ES, Shevach EM, Frank MM, Green I. Leukemic reticuloendotheliosis: presence of a receptor for cytophilic antibody. *Am J Med* 1974;**57**:108-14.
- ¹⁵ McKeever PE, Gravin AJ, Spicer SS. Immune complex receptors on cell surfaces. I. Ultrastructural demonstration on macrophages. *J Histochem Cytochem* 1976;**24**:948-55.
- ¹⁶ Rosenthal SH, Cassidy JT, Soderstrom S. Surface immunoglobulin on human peripheral lymphocytes: permanent preparations stained with horseradish peroxidase-labelled antibody. *J Lab Clin Med* 1974;**83**:564-90.
- ¹⁷ Warnke R. Alteration of immunoglobulin-bearing lymphoma cells by fixation. *J Histochem Cytochem* 1979;**27**:1195-6.
- ¹⁸ Mason DY, Bell JR, Christenson B, Biberfeld P. An immunohistological study of human lymphoma. *Clin Exp Immunol* 1980;**40**:235-48.
- ¹⁹ Dighiero G, Follezu JY, Roison JP, Ternynck T, Binet JL. Comparison of normal and chronic lymphocytic leukaemia lymphocyte surface Ig determinants using peroxidase-labelled antibodies. II. Quantification of light chains determinant in atypical lymphocytic leukemia. *Blood* 1976;**48**:559-66.
- ²⁰ Taylor BB, Duffus NP, Raff MC, De Petris S. Redistribution and pinocytosis of lymphocyte surface immunoglobulin molecules induced by anti-immunoglobulin antibody. *Nature (New Biology)* 1971;**233**:225-7.
- ²¹ Breton Gorius J, Gourdin MF, Reyes F. Ultrastructure of the leukemic cell. In: Catovsky D, ed. *The leukemic cell*. Edinburgh: Churchill Livingstone, 1981:87-128.

Requests for reprints to: Dr F Reyes, Inserm U.91, Hôpital Henri Mondor, 94010 Creteil, France.