Method for the simultaneous labelling of terminal deoxynucleotidyl transferase (TdT) and membrane antigens

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SUMMARY A method for the simultaneous labelling of terminal deoxynucleotidyl transferase (TdT) and membrane antigens is described. TdT is visualised in the cell nucleus with the peroxidase-antiperoxidase (PAP) method, and the immunogold method is used in combination with monoclonal antibodies against membrane antigens. The morphology of the labelled cells is well preserved for analysis by light microscopy and the preparations obtained can be kept permanently. This method is useful for the analysis of mixed cell proliferations, particularly in leukaemias.

Microscopical observations that depend on two immunological markers and the morphological appearances of cells are important for the comparison of leukaemic cells with corresponding normal cell types.¹ In acute leukaemia the most useful combinations involve the staining of the intracellular enzyme terminal deoxynucleotidyl transferase (TdT)² and one membrane antigen, such as the common acute lymphoblastic leukaemia (ALL) or HLA-DR antigens, which are usually performed by immunofluorescence methods.¹

We report here a technique that combines the immunogold method³ for the detection of membrane antigens with a peroxidase-antiperoxidase method (PAP)⁴ for the demonstration of TdT and permits analysis by light microscopy. The advantages of this method are: (a) the observations are made on conventional microscopes; (b) the cell preparations are permanent; and (c) the morphological detail is greater than with immunofluorescence preparations, hence facilitating the comparison of morphological characteristics with the immunologic phenotype of individual cells.

Material and methods

CELLS AND ANTIBODIES

The antibodies used and their specificities, sources, titres, and volumes applied are detailed in Table 1.

To set up the technique, which is referred to as the combined technique, the NALM-1 cell line¹¹ was used. This cell line, derived from a lymphoid blast crisis of chronic granulocytic leukaemia, expresses TdT, the common ALL antigen, and Ia-like determinants. The procedure was then tested in samples from six patients with leukaemia, whose diagnoses are listed in Table 2.

COMBINED TECHNIQUE

Cell separation

The mononuclear cell fraction from leukaemic samples (peripheral blood or bone marrow) was isolated by centrifugation at 400 g for 30 min on Lymphoprep (Nyegaard, Oslo, Norway). The cells were then washed, resuspended in buffer (phosphate buffered saline (PBS), 1% bovine serum albumin, 0.2% sodium azide, pH 7.4), and counted.

IMMUNOGOLD FOR SURFACE ANTIGENS

In each test a pellet of 10^6 cells was incubated with the relevant monoclonal antibody in the dilutions and volumes detailed in Table 1 for 30 min at room temperature. After washing, 40 μ l of neat immunogold was added to the pellet and incubated for 60 min at room temperature. The cells were then washed and cytocentrifuge preparations made with 2×10^5 cells per slide (Shandon Cytospin, 300 rpm, 7 min). It is convenient to mark the cell area with a diamond pen. At this stage the slides were dried and either stored at room temperature for some days

Table 1 Immunological reagents

Antibody	Specificity	Source	Titre	Volume	Reference
J5	Human common acute lymphoblastic leukaemia antigen	Coulter Electronics	Neat	5 μ l/10 ⁶ cells	5
OK1a	Human HLA-DR framework	Ortho, Raritan, New Jersey, USA	Neat	5 μ l/10 ⁶ cells	6
RFB1	Immature human haemopoietic cells and T lineage cells	Dr MP Bodger Department of Immunology, Royal Free Hospital, London	1/100 of ascitic fluid	50 μ l/10 ⁶ cells	7
FMC 8	Differentiation antigen expressed in some human blood cells	Dr H Zola Flinders Medical Center, Bedford Park, South Australia	Supernatant as supplied	50 μ l/10 ⁶ cells	8
FMC 25	Platelet membrane glycoprotein Ib	Dr H Zola	Supernatant as supplied	50 μ l/10 ⁶ cells	9
Immunogold	Goat antibodies to mouse immunoglobulin linked to 40nm colloidal gold particles	Janssen Life Sciences Products, Beerse, Belgium	Neat	40 μ l/10 ⁶ cells	3
Anti-TdT	Rabbit antiserum to calf TdT	Professor FJ Bollum Bethesda, Maryland, USA	1/10	10 μ l per slide (approx 2 $\times$ 10 ⁶ cells)	10
Goat antirabbit PAP complex	Antirabbit IgG Rabbit antiperoxidase complexed with horseradish peroxidase	Miles Laboratories, Slough Bioproducts UCB, Brussels, Belgium	1/200 1/300	20 μ l per slide 20 μ l per slide	

Table 2 Comparison of the combined method with conventional immunofluorescence methods in samples from patients with leukaemia

Case	Diagnosis	Percentage of positive cells*					
		TdT	J5	OK1a	RFB1	FMC8	FMC25
1	Mixed BC Myeloblastic + lymphoid + megakaryoblastic	10 (10)		65 (70)		42 (50)	25 (33)
2	Lymphoid BC	69 (94)	98 (93)	56 (62)		100 (95)	
3	Mixed BC Lymphoid + myeloblastic	64 (63)	10 (13)	80 (64)	73 (90)		
4	Lymphoid BC	90 (86)	97 (95)	100 (82)			
5	Mixed acute leukaemia Lymphoid + myeloblastic	80 (70)	6 (8)	25 (60)	92 (98)		
6	c-ALL	88 (92)	62 (96)				

BC = blast crisis of chronic granulocytic leukaemia.

c-ALL = common acute lymphoblastic leukaemia.

*Percentages for combined method (immunofluorescence results in brackets).

(maximum one week) or processed immediately for TdT.

PAP technique for TdT

Fixation was done in methanol at 4°C for 45 min. After fixation, slides were washed in PBS for 10 min in a container with buffer and a magnetic stirrer. The cells were incubated successively with anti-TdT, goat antirabbit immunoglobulin, and PAP complex. The 30 min incubations were done at room temperature in a moist chamber and each was followed by washes in PBS.

After the last wash, slides were briefly immersed in Tris-HCl buffer (0.05 M Tris-HCl 0.9% NaCl, pH 7.4) and peroxidase activity was developed by immersion for 10 min at room temperature in the dark in diaminobenzidine medium: 0.3 mg/ml diaminobenzidine (Aldrich Chemical Co, Gillingham, UK), 0.03% H₂O₂ in Tris-HCl buffer.

Counterstaining and mounting

After washing with Tris-HCl buffer and a brief immersion in distilled water, the cells were counterstained with 1% methyl green (5-10 min), washed in water, dehydrated by immersion in graded ethanols (50, 70, 90, absolute ethanol), cleared with xylene, and mounted permanently with DPX (Raymond Lamb, London). The preparations were examined with a conventional light microscope.

Endogenous peroxidase

Endogenous peroxidase could be blocked by immersion in 0.3% H₂O₂ in methanol for 30 min immediately after fixation.

Controls

For negative controls either the monoclonal antibody in the first step, the anti-TdT in the second, or both were omitted. We tested the samples by con-

Fig. 1 *Nalm-1 cells. TdT detection by the peroxidase-antiperoxidase method. The reaction is present mainly in the nucleus. A dividing cell shows the reaction diffusely distributed in the cytoplasm, but absent in the area containing the chromosomes (arrow). $\times 1000$.*

Fig. 2 *Case 3, combined method: TdT and J5. Large blasts are TdT negative, J5 negative (large arrow); medium size blasts TdT positive, predominantly J5 negative (small arrow); and small blasts TdT positive, predominantly J5 positive (arrow head). TdT reaction is stronger in small blasts. Original magnification $\times 1000$.*

ventional immunofluorescence for TdT and the relevant surface antigens. Table 2 shows a comparison of the results with those obtained by the new method.

Results

The percentage of positive cells by the combined technique correlated well with the results obtained by conventional immunofluorescence methods with similar reagents (Table 2). TdT was detected as a brown reaction present in the nucleus in a fine particular pattern. The intensity of reaction varied from cell to cell. In some cells a weak reaction was also present in the cytoplasm. In dividing cells the reaction was diffusely distributed through the cytoplasm, with the area containing the chromosomes devoid of reactivity (Fig. 1). In leukaemic samples the TdT reaction tended to be stronger in small blasts with a lymphoid appearance (Fig. 2). Inhibition of endogenous peroxidase with H_2O_2 weakened the TdT reaction. When this procedure was not used rare erythrocytes and myeloid cells were positive; this did not constitute a problem because they could be recognised morphologically. Cells reacting with the monoclonal antibodies had numerous black dots around the surface membrane. In the strongly reactive cells these dots were confluent and covered variable sections of the cell surface as a black lining (Fig. 3). Most of the unreactive cells had no dots. Cells with fewer than three dots were considered negative. Ultrastructural studies (results not shown) have shown that the individual dots correspond to patches of 40 nm colloidal gold particles.¹²

The combined method was particularly useful for analysing the cellular components of leukaemias with different types of blasts (cases 1, 3, 5), characteristically seen in mixed acute leukaemias and mixed blast crisis of chronic granulocytic leukaemia. In case 1 TdT positive blasts also expressed the antigens recognised by OKla and FMC8, but the antiplatelet antibody FMC25 reacted only with TdT negative blasts. In cases 3 and 5 with mixtures of myeloblasts and lymphoblasts large blasts were TdT negative, J5 negative, and RFBI positive; medium size blasts were TdT positive, predominantly J5 negative, and RFBI positive; and small cells were TdT positive, predominantly J5 positive, and RFBI positive (Figs. 2 and 3). In the control preparations all the cells were negative for the omitted antibody. The percentage of positive cells was the same in experiments where the two steps of the technique were performed separately and in combination, which shows that they do not interfere with each other.

Fig. 3 Case 5, combined method: TdT and RFBI. One large blast is RFBI positive, TdT negative (arrow), the other blasts express both markers. Original magnification $\times 1000$.

Discussion

Although more time consuming than conventional immunofluorescence methods this technique requires only the use of a simple light microscope and provides permanent preparations. We found a good correlation between the combined technique and conventional immunofluorescence methods both for TdT and surface antigens, and the specificity of both components of the method was also good as a positive reaction in the controls was never seen. The morphological detail obtained was good and from each single cell it was possible to obtain three types of information: morphology and expression of TdT or of a membrane antigen, or both.

This method is particularly useful in the study of mixed leukaemias and blast crisis of chronic granulocytic leukaemia, where frequently two or more different populations of blasts coexist.¹³ In these cases it is possible: (a) to analyse the different morphological characteristics of the blasts that correspond to each of the immunological phenotypes present (cases 3 and 5), and (b) to determine if TdT and a membrane marker can be simultaneously present in the same cell or are always expressed in different blasts (case 1). This technique can be used for the analysis of single cells in certain tissues—for example, cerebrospinal fluid and remission bone marrow—where it may help identify cells with a leukaemic membrane phenotype and thus distinguish them from putative normal precursors.¹ Another possible area of application could be in the studies of *in vitro* differentiation of lymphoid cell lines, where changes in the expression of two markers could be

correlated with the morphological evolution.¹⁴

In the present work we studied the expression of five different surface antigens: the same approach could be extended to any cell membrane antigen detectable by a monoclonal antibody or heteroantiserum. A possible variant of the technique is the detection of cytoplasmic immunoglobulin¹⁵ instead of TdT and its association with the membrane phenotype as shown by the immunogold technique. The combination of the immunogold technique with cytochemistry has been reported from this laboratory both in studies by light microscopy¹⁶ and by electron microscopy.¹²

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