Modified immunocytochemical slide technique for demonstrating surface antigens on viable cells

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SUMMARY Further developments of an immunoenzymatic slide technique to demonstrate cell surface antigens with monoclonal antibodies are described. In this method cells are attached to polylysine coated glass slides in order to facilitate the handling of low cell numbers and to save antibodies and time for washing the cells. The technique has been modified for the labelling of viable cells. Endogenous peroxidase is used as an additional cell marker which does not interfere with the demonstration of antigens on the cell surface by immunoperoxidase methods. Damaged cells can be identified reliably, thereby minimising interpretation errors due to non-specific antibody uptake. A double labelling technique employing peroxidase and alkaline phosphatase coupled reagents is presented. Results of this slide technique are clear cut, so that evaluation can be performed by trained technicians.

Immunocytochemistry has become a widely used procedure for characterising cells in tissue sections and in suspension. The introduction of monoclonal antibodies¹ substantially increased the specificity, reliability, and reproducibility of this technique. Cell typing with monoclonal antibodies has led to the discovery of heterogeneity within morphological homogeneous cell populations. This has not only resulted in a new understanding of cell physiology but also has important consequences for the diagnosis and treatment of human disorders. Lymphocytic leukaemias and non-Hodgkin's lymphomas, for example, can now be classified by the finding of intracellular and surface antigens, and the resulting subgroups are of major clinical importance.²⁻⁴

Antibodies bound to antigens on cells in suspension can be detected by cytotoxicity techniques or by linking a radioactive isotope, a fluorescent dye, an enzyme, or particles like *Staphylococcus aureus* or gold to the antibody itself or to secondary antibodies. Immunoenzymatic techniques have become particularly popular since the description of highly sensitive methods such as the peroxidase-antiperoxidase technique⁵ or the avidin-biotin complex technique.^{6,7} In this paper we describe an immunoenzymatic slide technique, which is based

on a procedure previously described by one of the authors.⁸ In this method cells are attached to polylysine coated glass slides in order to facilitate the handling of low cell numbers and to save antibodies and time for washing the cells. The test system has been modified for labelling viable cells and eliminates problems due to endogenous peroxidase activity without destroying antigens or employing non-peroxidase enzymes. A double staining technique is described.

Material and methods

CELLS AND ANTIBODIES

Peripheral blood and bone marrow mononuclear cells were isolated by Ficoll-Metrizoate density gradient centrifugation (Lymphoprep, Nyegaard, Oslo, Norway). The cells were washed three times in phosphate buffered saline (PBS) and resuspended in the same buffer at $0.5-2.0 \times 10^6/\text{ml}$.

The following antibodies were used: OKT11, OKT4, OKT8, OKT9, OKT10 (Ortho); Leu1, Leu7, Leu8 (Becton Dickinson); B1, I2 (Coulter); W6/32HK (kindly provided by Dr Ziegler, Tübingen) and polyclonal mouse IgG and IgM (Coulter), used as controls for non-specific binding of the monoclonal antibodies; biotinylated horse anti-mouse IgG, avidin DH and biotinylated horseradish peroxidase (Vector), antimouse subclass specific

antisera produced in rabbit (Zymed) and goat (Meloy); swine antirabbit IgG and rabbit peroxidase-antiperoxidase complex (Dako); biotinylated swine antigoat IgG (Tago); alkaline phosphatase coupled avidin (Sigma). Unless stated otherwise, antibodies and other reagents were diluted in PBS containing gelatin (0.1%) and serum (5%). Human or rabbit serum was used for single labelling, and swine serum was used for double labelling. After each incubation step the cells were washed with PBS in a staining jar or separately by dropwise addition of buffer and removal by suction. Fixation, incubation (15 min in a moist chamber), and washing (5 min) were performed at room temperature throughout the procedure.

METHOD

Preparation of slides

Small drops of a gum and sucrose solution (20 g of gummi arabicum, 20 g of sucrose, and 0.13 g of NaN_3 in H_2O) were placed on clean glass slides. After drying overnight the slides were carefully wiped with a cloth soaked in a polysiloxane solution (84 ml of propanol, 1 ml of concentrated H_2SO_4 , and 15 ml of dimethyl-polysiloxane (Sigma), ready for use if cleared after shaking). After drying, at least overnight, the gum and sucrose solution was removed by running tap water and 5% Extran (Merck). Drops of polylysine (MW 15 000–30 000, Sigma), 0.5 mg/ml in PBS, were applied to the resulting small spots, separated by water repellent polysiloxane. After incubation for 30 min in a moist chamber the slides, if not used immediately, were frozen at -20°C .

Attachment of cells

After thawing and washing the slides with PBS, 50 μl of the cell suspension ($0.25\text{--}1.0 \times 10^5$ cells in serum free PBS) was deposited by pipette on to each polylysine coated spot. The cells were allowed to adhere to the positively charged glass surface for 15 min. After rinsing the slides with PBS, any free polylysine was blocked by the addition of a drop of a mixture of PBS, gelatin (0.1%), and human serum (5%) containing 20 mM NaN_3 for 5 min.

Single labelling technique

- 1 Blocking buffer was sucked off and 10 μl of monoclonal antibodies (usually diluted 1/50–1/400) was added to the cells. NaN_3 (20 mM) was included in the diluting buffer to prevent capping and shedding by the viable cells.
- 2 Staining of dead cells by the addition of a drop of trypan blue (0.1% in 0.9% NaCl, 20 mM NaN_3).
- 3 Fixation (0.05% glutaraldehyde in PBS, 15 min).

- 4 Staining of endogenous peroxidase with 4-chloro-1-naphthol for 20 min (100 mg of 4-chloro-1-naphthol (Sigma) in 2.0 ml of absolute ethanol and 500 ml of 0.05 M Tris-HCl buffer, pH 7.6, containing 3.8 g of NaCl; stirred at room temperature overnight, filtered, and stored in aliquots at -20°C ; H_2O_2 was added to a final concentration of 0.06% immediately before use).

- 5 Biotinylated horse antimouse IgG (1/200).

- 6 Avidin-biotin-peroxidase complex prepared 30 min before use according to the recommendations of the manufacturer (1/100 each).

- 7 Staining of marker peroxidase with 3-amino-9-ethyl carbazole for 10 min (20 mg of 3-amino-9-ethyl carbazole (Sigma) in 5.0 ml of N, N-dimethyl formamide (Sigma) plus 100 ml of 0.1 M acetate buffer, pH 5.2; stirred at room temperature, filtered, and stored in aliquots at -20°C ; H_2O_2 was added to a final concentration of 0.0006% immediately before use).

- 8 Mounting in Kaiser's glycerine jelly (Merck) without counterstaining.

Double labelling technique

Blocking of polylysine, incubations, washings, fixation, and staining with trypan blue, 4-chloro-1-naphthol, and 3-amino-9-ethyl carbazole were carried out as described for the single labelling technique.

- 1 Incubation of the cells with a mixture of two primary antibodies (A and B) of different species—for example, goat and rabbit—or different isotypes.

- 2 Trypan blue staining.

- 3 Fixation.

- 4 In the case of primary antibodies of different isotype, addition of a mixture of appropriate dilutions of two secondary antibodies (goat anti-A and rabbit anti-B), selected for maximal reactivity with the respective isotype without cross reactivity with other isotypes.

- 5 4-Chloro-1-naphthol.

- 6 Addition of a mixture of two species specific secondary/tertiary antibodies (swine antigoat, biotinylated, and swine antirabbit, unlabelled, 1/20 each).

- 7 Incubation with a mixture of avidin, alkaline phosphatase coupled, and rabbit peroxidase-antiperoxidase complex, 1/100 each.

- 8 Demonstration of the alkaline phosphatase activity with one of the following substrates: fast blue BB, 1 mg/ml, and naphthol-AS-MX-phosphate, 1 mg/ml, or variamine blue B, 1 mg/ml, and naphthol-AS-GR-phosphate, 1 mg/ml, in 0.2 M Tris-HCl buffer, pH 8.7. Stock solutions of the substrates and couplers were prepared in N, N-dimethylformamide at a concentration of

Fig. 1 Identification of B lymphocytes (three red stained cells) in a normal bone marrow aspirate. The red 3-amino-9 ethyl carbazole substrate contrasts well with the black-brown 4-chloro-1-naphthol, developed by the endogenous peroxidase of granulopoiesis or pseudoperoxidase of haemoglobin containing erythroid cells. Original magnification $\times 800$.

Fig. 2 Double labelling of peripheral blood mononuclear cells with OKT8 (peroxidase, red cells) and Leu7 (alkaline phosphatase, one blue cell). There is one cell with both markers on its membrane, stained black-purple. Monocytes can be identified by their black-brown perinuclear deposits of 4-chloro-1 naphthol, developed by their endogenous peroxidase. Original magnification $\times 800$.

50 mg/ml. 1 mM levamisole was added to the incubation medium to inhibit endogenous alkaline phosphatase* (all reagents from Sigma).

9 Demonstration of the peroxidase activity with 3-amino-9-ethyl carbazole.

Evaluation

At least 200 cells were evaluated (peroxidase, red; alkaline phosphatase, blue or green; both enzymes on the same cell, mixed colours). Monocytes and granulocytic cells were identified by their endogenous peroxidase activity (black-brown intracellular deposits). Cytocentrifuge preparations of the cell suspension analysed were prepared for morphological cell examination and for control of any cell loss during the labelling procedure.

Results

DEVELOPMENT OF THE TECHNIQUE

The original slide technique has been modified to facilitate the surface marker analysis of cells in suspension.

1 It was useful to label viable cells with the primary antibodies, followed by the addition of trypan blue. In this way antigens are not destroyed by fixation and damaged cells, which, in addition, do not adhere to polylysine as effectively as viable cells, can be identified by trypan blue uptake.

2 After the addition of monoclonal antibodies the cells are fixed in order to attach them firmly to the glass surface during the following incubation steps and to block antibody uptake by Fc receptors. Fixation with even higher concentrations of glutaraldehyde (up to about 0.3%) does not appreciably impair the antigenicity of the monoclonal antibodies previously bound to the cell surface.

3 Instead of inhibiting endogenous peroxidase by various techniques^{10,11} or using non-peroxidase enzymes, the endogenous enzyme is used as an additional cell marker: after incubation of the fixed cells

with 4-chloro-1-naphthol before the addition of the marker peroxidase, peroxidase positive cells can be identified by black-brown deposits in the cytoplasm, which contrast well with the bright red granules produced later on by the horseradish peroxidase and 3-amino-9-ethyl carbazole on the surface of the same cell or other cell populations (Fig. 1).

4 Finally, a double marker system was established for use with the slide method. Alkaline phosphatase was chosen as the second enzyme, since there is a wide selection of substrates and couplers from which to choose the one giving the best contrast to the red peroxidase substrate.¹² Naphthol-AS-MX-phosphate/fast blue BB (blue) and naphthol-AS-GR-phosphate/varianine blue B (green) gave the best results among several other combinations tested. To link the enzymes to antibodies of different species or different isotypes of the same species, a combination of the peroxidase-antiperoxidase method and a biotin-avidin system proved superior to enzymes directly coupled with the secondary antibodies.

APPLICATION FOR MARKER ANALYSIS

To demonstrate the validity of the method described, peripheral blood mononuclear cells from 27 normal volunteers (age 19–67 years, median 29) were analysed with monoclonal antibodies of known specificity. The Table shows that the values obtained by our technique compare well with those in the published work. Except for Leu7 and Leu8, percentages of the slide technique are higher than those of fluorescence techniques because they represent lymphoid cells, excluding monocytes by their endogenous peroxidase activity. Values for Leu7 and Leu8 positive cells are low compared with previous reports, possibly due to a sampling error since there is a wide range of normality for both markers.^{13–15} Percentages of 12 positive cells reported in the published work are misleading since they include 1a positive monocytes. Using the slide technique, 12

Reactivity of monoclonal antibodies with peripheral blood lymphocytes of normal donors using the slide technique compared with reference values reported in the published work using immunofluorescence techniques

Antibody	% positive cells Slide technique		Reference values Immunofluorescence	Reference no
OKT11	79.9 ± 7.8	(27)*	74.0 ± 7.0	(?)* 13
Leu1	67.5 ± 9.2	(17)	60.0 ± 1.0†	(22) 14
OKT4	47.9 ± 8.8	(27)	39.0 ± 1.0†	(37) 14
OKT8	25.6 ± 6.8	(27)	19.5 ± 0.7†	(38) 14
Leu7	7.4 ± 3.6	(18)	11.0 ± 4.0†	(38) 14
Leu8	58.1 ± 12.4	(16)	68.0 ± 7.0	(?) 15
B1	14.3 ± 8.0	(27)	9.1 ± 2.0	(13) 16
I2	14.7 ± 7.9	(22)	(see text)	17
OKT10	7.5 ± 6.8	(17)	5.8 ± 2.5	(16) 18
T4/T8	1.95 ± 0.56	(27)		

*Number of samples analysed.

Values given as mean ± SD or SEM†.

positive, endogenous peroxidase negative cells are strongly reactive B cells and some weakly positive activated T cells and null cells.¹⁷

Discussion

The purpose of this paper is to present further developments of an immunoenzymatic slide technique previously described by one of the authors.⁸ The advantages of this slide technique have already been discussed: easy handling of the cells without time consuming centrifugation steps and reduction of the amount of cells ($0.25\text{--}1.0 \times 10^5$ cells/test), expensive reagents ($10 \mu\text{l}$), as well as the space to store the preparations.

Results of surface marker analysis with monoclonal antibodies and a fluorescence method, based on the same slide technique, have recently been published.¹⁹ Enzyme coupled reagents were used since there are definite practical advantages—for example, permanent preparations and evaluation by an ordinary light microscope—and the sensitivity is at least comparable to immunofluorescence. The assay is particularly sensitive if unlabelled antibody techniques such as peroxidase-antiperoxidase⁶ and alkaline phosphatase-anti-alkaline phosphatase²⁰ or the avidin-biotin-peroxidase complex technique⁷ are used instead of enzyme-antibody conjugates. Increased sensitivity has, however, to be balanced against the additional expenditure of time, compared with directly coupled reagents which are now commercially available in high quality.

In our assay horseradish peroxidase can be used for immunoenzymatic labelling of peroxidase positive cells without inhibiting the endogenous enzyme: both peroxidases can be visualised separately by the successive development of different substrates, resulting in visible precipitates at the site of either enzyme.²¹ The lysosomal enzyme in monocytes and granulocytes and the pseudoperoxidase activity in erythroblasts can be used as an additional cell marker, which does not interfere with subsequent surface marker analysis. Reliable discrimination of the two labels even within a single cell is due to localisation on different cell structures—that is, granules or cytoplasm and surface membrane. The same holds true for our double labelling assay: if peroxidase and alkaline phosphatase are bound to different cells or different structures within a single cell—for example, TdT in the nucleus and Ia on the cell membrane, data not shown—the resulting coloured precipitates can be reliably discriminated. Double labelling of antigens on the same cell structure, however—for example, OKT8 and Leu7 on the membrane of a single cell (Fig. 2)—results in mixed colours which cannot always be definitely

identified.^{12,22,23} Up to now, immunofluorescence using different fluorochromes—for example, fluorescein isothiocyanate and rhodamine isothiocyanate—has been found more appropriate.²⁴

Labelling of viable cells was chosen to avoid antigen denaturation by fixation and to obtain comparable results to flow cytometry and reliable predictions of sorting experiments. The use of viable cells introduces some sources of error which can, however, be overcome by careful controls: binding of the primary antibody (non-F(ab)₂ preparations) to Fc receptors can be minimised by blocking of receptor sites with normal serum and checking residual binding by non-immune antibodies of the same isotype. Non-specific binding of antibodies by damaged cells gives a more homogeneous labelling pattern and can be shown by trypan blue uptake by the respective cells in our assay. Capping and shedding of antigens can be inhibited by the addition of NaN_3 during the first incubation and limiting the incubation time to 15 min. Cell loss was negligible if the slides were carefully prepared as described in the Material and methods section and any protein removed by washing the cells in PBS. Nevertheless, any cell loss should be excluded by preparing cytocentrifuge slides of the same cell suspension and comparing the composition of the two preparations by conventional morphology and cytochemistry. To minimise cell loss the cells are fixed with 0.05% glutaraldehyde after incubation with the primary antibody, thereby linking them to polylysine without destroying the antibody or inactivating endogenous peroxidase. Equally important is a further blocking of Fc receptors by glutaraldehyde, since non-specific binding of polyclonal antibodies during the following incubation steps occurs even more readily than with monoclonal antibodies.

The immunoenzymatic slide technique, as described in this paper, has now been used regularly for two years. Surface marker analysis of cells from peripheral blood, bone marrow, cerebrospinal fluid, effusions, fine needle aspirates, and cell lines can be performed without any problems, as long as single cell suspensions are prepared (cell aggregates do not stick to the polylysine firmly and will therefore be lost during the labelling process). The technique was particularly useful for the analysis of bone marrow transplants after “purging” with anti-T cell antibodies and complement, since damaged cells, still binding the antibodies, can be discriminated from viable T cells. As in flow cytometry, all monoclonal and polyclonal antibodies which bind to the surface of viable cells can be applied—for example, for leukaemia and lymphoma typing, lymphocyte subset analysis in infectious or inflammatory diseases or immune deficiency states. Intracellular antigens can

also be shown using a modification of the technique described (unpublished observations). Since endogenous peroxidase positive and damaged cells can easily be identified, the evaluation can be performed by a technician. Furthermore, automated cell counting should be feasible using pattern recognition systems such as those which have been developed for differential leucocyte counting. This slide method is a useful alternative to currently available techniques, especially if only a few cells have to be analysed with expensive reagents.

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