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Simple immunocytochemical staining procedure for lymphoid cell surface markers done on cell smears

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The value of monoclonal antibodies as probes for leukaemic heterogeneity and haemopoietic differentiation is well recognised.¹ We report a simple indirect immunoperoxidase staining method to demonstrate various surface antigens on smears of lymphocytes and lymphoblasts using commercially available monoclonal antibodies.

Material and methods

The mononuclear cell fraction of edetic acid or heparin anticoagulated venous or marrow blood was obtained by centrifuging on Ficoll-Paque (Pharmacia, Sweden).² The mononuclear cells were smeared on to clean glass slides. Smears were dried thoroughly in air and fixed for 30 s in 4°C buffered formalin-acetone, pH 6.6 (20 mg Na₂HPO₄, 100 mg KH₂PO₄, 30 ml H₂O, 45 ml acetone, and 25 ml formalin).³ The fixed smears were then washed immediately with two changes of phosphate buffered saline (PBS), pH 7.0-7.2, for 5 min each. The subsequent procedure was similar to that described by McMillan *et al.*⁴ Primary monoclonal mouse anti-human antibodies (Coulter Clone, USA) diluted in PBS were spread on to the smears. The antibodies and the dilutions used were: T11 (pan-T)-1/10; I2 (anti-HLA-DR)-1/15; B1 (pan-B)-1/10; T4 (I-helper)-1/5; T8 (T-suppressor)-1/5; J5 (anti-cALL)-1/10. Smears were incubated for 30 min in a moist chamber, and then washed twice in PBS for 5 min. Horseradish peroxidase conjugated goat anti-mouse IgG (TAGO, USA), diluted 1/15 in PBS was spread on to the smears, which were then incubated for 15 min in a moist chamber. The smears were washed again in PBS, twice for 5 min. The enzyme colour was developed in H₂O₂/3-amino-9-ethyl carbazole.⁵ The smears were then counterstained in Mayer's haematoxylin for 10 min, rinsed in running tap water for 10 min, fan dried, and cover slipped with Kaiser's glycerin jelly.

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Results and discussion

Under the light microscope, positively stained cells showed a reddish brown rim encircling the cell (Figure). Staining was stronger on the thicker part of a smear, and hence the optimum area for examination was the middle third of the smear, where strong staining and good cell morphology could be seen.

The separation of the mononuclear cells from the erythrocytes and granulocytes serves two purposes. Firstly, it concentrates the mononuclear cells and, secondly, it removes cells containing endogenous peroxidase activity from the blood. Fifty microlitres of diluted antibody is sufficient to form a thin film covering a smear, and care should be taken to drain off excess PBS before the application of antibodies to avoid over dilution. The mononuclear cells should be separated as soon as the sample is received to avoid cell degeneration, but once the mononuclear cell smears are prepared and dried storage at room temperature for up to four days will not cause any change in antigenicity.

The monoclonal antibodies that are currently used in our laboratory come from Coulter and we

Common acute lymphoblastic leukaemia. Lymphoblasts show a positive surface staining for J5 (anti-cALL). An unstained lymphocyte (arrow) is also seen (indirect immunoperoxidase, $\times 1000$).

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found that all the antibodies mentioned above showed equally good staining results on our smears. In normal adults the number of peripheral blood lymphocytes stained by these markers were as follows: T11: 75–90%; B1: 10–20%; I2: 10–20%; T4: 55–65%; T8: 25–35%; J5: 100% negative. These figures are in close agreement with those given by the manufacturer when using immunofluorescence.

Using this method we have successfully demonstrated various surface markers on normal lymphocytes and leukaemic cells in acute lymphoblastic leukaemia, lymphoma, and chronic lymphocytic leukaemia. We are aware that the immunofluorescence method is equally good for demonstrating cellular antigens on smeared cells, and a report describing such technique has recently been published in the *Journal of Clinical Pathology*.⁷ The advantage of the method described in the present paper over immunofluorescence is that a simultaneous study of morphology and immunocytochemistry is possible and the stained smears can be filed as a permanent record.

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Fluorimetric method for measuring chemotaxis

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Polymorphonuclear leucocyte chemotaxis is a fascinating phenomenon and its molecular, biological, and clinical aspects have been widely studied. Most investigators have used a variation of the test developed by Boyden.¹ The chief disadvantage of this test is that it requires the chemotactic response to be measured microscopically on stained filters, either by cell counting or by measuring the distance travelled into the filter. Gallin *et al*² tagged granulocytes with ⁵¹Cr and measured the response by counting the radioactivity present on collecting filters with a pore size impermeable to leucocytes. The labelling step increases the duration of the experiment and the hazard of cell damage.

We have also used a second filter to collect migrating cells but have developed a chemical method for counting them. The method assays for DNA as described by Kapuscinski and Skoczylas.³ The natural fluorescence of the dye 4', 6-diamidino-2-phenylindole (DAPI) is enhanced

when complexed to DNA. The fluorescence enhancement is linearly proportional to the concentration of DNA at appropriate DAPI:DNA ratios, even in the presence of protein and RNA. By means of a standard curve, changes in fluorescence can be read directly as cell numbers.

Material and methods

Polymorphonuclear leucocytes were purified from venous blood using the method described by Boyum.⁴ Contaminating red cells were removed by hypotonic lysis. The polymorphonuclear leucocytes were suspended at a concentration of 1×10^6 /ml in medium 199 containing 0.9% glucose and 1.75% sodium bicarbonate and applied to 8 μ m and 0.45 μ m cellulose nitrate filters (Millipore) sandwiched in Teflon Boyden chambers. The chemoattractant was 0.5% casein in medium 199. The chambers were incubated in 5% CO₂ at 37°C for 90 min. For some experiments cell concentrations and incubation times were varied. The variables were tested in duplicate. After incubation, two 0.45 μ m filters were removed from the chambers, placed in 1 ml of 5 mM Hepes buffer containing 12 mM NaCl, and sonicated with a Dawe Soniprobe for 20 s. One millilitre of DAPI at an appropriate concentration in Hepes buffer was added to each vial, mixed by inversion, and the fluorescence measured on a Perkin-Elmer 204 spectrophluorimeter. Filters from chambers containing casein but no cells were used as