Technical methods

Single incubation double esterase cytochemical reaction using a single coupling reagent

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Since 1953, when Gomori¹ reported on the use of chloroacyl esters as histochemical substrates, esterase cytochemistry has come to play an important part in the identification and classification of the acute myeloid leukaemias.^{2,3} The substrate specificities of normal granulocytes (naphthol AS-D chloroacetate) and normal monocytes (α -naphthyl butyrate)⁴ now form the basis of identifying poorly differentiated myeloid leukaemia cells as committed to the granulocytic or monocytic lineages. Methods for combining two esterase stains on a single slide^{3,5} have enabled the simultaneous visualisation of leukaemic cells exhibiting different substrate preferences. All methods previously described require the blood or marrow smear to be stained twice, using separate coupling reagents with each substrate—for example, fast blue BB with naphthol AS-D chloroacetate and fast garnet GBC with α -naphthyl butyrate.

Fast blue BB is an excellent coupling reagent when used with either naphthol AS-D chloroacetate or α -naphthyl butyrate as substrates. When naphthol AS-D chloroacetate is the substrate, the reaction product is bright blue; the reaction occurs predominantly in polymorphonuclear neutrophils and their precursors and only minimally in monocytes. In sharp contrast, when α -naphthyl butyrate is the substrate the reaction product is dark brown; the reaction occurs predominantly in monocytes and their precursors and as dot like positivity in a variable number of T lymphocytes.⁶ This difference has allowed the development of a single incubation medium containing naphthol AS-D chloroacetate, α -naphthyl butyrate, and fast blue BB, which provides the same information with similar sensitivity to the traditional sequential staining methods used in performing "double esterase" stains.

Material and methods

MATERIAL

Buffered formalin/acetone (20 mg Na₂HPO₄, 100 mg KH₂PO₄, 30 ml distilled water, 45 ml acetone, 25 ml concentrated formalin)
50 ml 0.1 mol/l phosphate buffer pH 8.0
2.5 mg naphthol AS-D chloroacetate (Sigma no N-0758) in 1 ml acetone
4 mg (4 μ l) α -naphthyl butyrate (Sigma no N-8000) in 1 ml acetone
80 mg fast blue BB salt (Gurr/BDH no 34177).

METHOD

- 1 Fix air dried smears in buffered formalin/acetone for 30 s.
- 2 Wash briefly in distilled water and air dry.
- 3 Mix the fast blue BB vigorously with the buffer.
- 4 Add 1 ml of acetone to the naphthol AS-D chloroacetate, agitate until dissolved, and mix with the buffer/fast blue BB.
- 5 Add the α -naphthyl butyrate to 1 ml of acetone, agitate until dispersed, and mix with the buffer/fast blue BB/naphthol AS-D chloroacetate.
- 6 Pour substrate solution into a Coplin jar in which the fixed slides have been placed and allow to incubate at room temperature for 15–30 min.
- 7 Wash in distilled water.
- 8 Counterstain with aqueous haematoxylin for 2–5 min.
- 9 Rinse in distilled water, air dry, and coverslip using aqueous mounting medium.

Steps 4 and 5 should be carried out as rapidly as possible. Step 7 should be carried out by running distilled water into the Coplin jar until the substrate solution (deep violet) has been cleared. The dark brown reaction product with α -naphthyl butyrate is soluble in organic solvents.

Results

Granulocytes and their normal and leukaemic precursors stain bright blue. Monocytes and their normal and leukaemic precursors stain dark brown. A proportion of normal T lymphocytes show localised dark brown positivity, as do a variable proportion of cells in T cell malignancies. The typical appearances in normal peripheral blood and bone marrow and in a variety of cases of acute myeloid leukaemia are shown in Figs. 1–6.

Fig. 1

Fig. 2

Fig. 3

Fig. 1 Normal peripheral blood buffy coat. Three neutrophils show blue chloroacetate positivity. Three monocytes show dark brown butyrate positivity. One lymphocyte shows localised dark brown butyrate positivity, the other is negative.

Fig. 2 Normal bone marrow. Six neutrophils and granulocyte precursors show blue chloroacetate positivity. One monocyte shows dark brown butyrate positivity. One lymphocyte and five normoblasts are negative.

Fig. 3 Acute myeloblastic leukaemia (M2) marrow smear. Eight cells show blue chloroacetate positivity. Three cells and a lymphocyte are negative.

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Fig. 4

Fig. 4 *Acute promyelocytic leukaemia (M3) buffy coat. Nine cells show localised or heavy overall blue chloroacetate positivity. Multiple Auer rods are present in two cells. The remainder show fine scattered blue chloroacetate granules.*

Fig. 5

Fig. 5 *Acute myelomonocytic leukaemia (M4) buffy coat. Ten blast cells show dark brown butyrate positivity. One blast cell shows blue chloroacetate positivity only and one shows a mixture of chloroacetate and butyrate positivity. The remaining cells are negative or show a fine scatter of blue chloroacetate granules.*

Fig. 6

Fig. 6 *Acute monoblastic leukaemia (M5) marrow smear. Twelve blast cells and promonocytes show dark brown butyrate positivity of varying intensity. One normoblast and the remaining leucocytes are negative.*

Phosphate buffer at pH 8.0 gave the sharpest staining reactions, although there was little difference at pH 7.0 or pH 7.5. As the buffer pH was increased above pH 8.0 staining with both substrates became progressively weaker, especially above pH 9.0. Below pH 7.0 staining with α -naphthyl butyrate became weaker, and below pH 5.0 staining with naphthol AS-D chloroacetate began to disappear.

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Simple technique to identify haemosiderin in immunoperoxidase stained sections

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Identifying iron compounds in immunoperoxidase stained sections usually presents little or no problem to the trained eye. The distinction between peroxidase positive staining and haemosiderin becomes important when one is in doubt as to which compound is giving the "brown positive" result.

Recent biopsies have presented this problem. Although naturally occurring endogenous peroxidase activity has been blocked in the sections there has been confusion between the brown staining of haemosiderin and the positive brown staining of the 3-3' diaminobenzidine tetrahydrochloride, especially in weakly positive sections.

The staining of serial sections with haematoxylin and eosin and by Perls' Prussian blue is routinely performed when this problem arises. But unless a comparator microscope is used to check the identity of the stained cell, the problem remains. Counterstaining the peroxidase stained slide with Perls' Prussian blue technique, however, permits the

identification of the two compounds on the same slide.

Material and methods

Sections were cut from biopsy specimens of surgically removed pituitary gland which had been embedded in paraffin and fixed in formalin. Ten 3 μ m serial sections were prepared and dried in 37°C incubator for 12 h.

The immunoperoxidase staining method used was an adaptation of the method of Kovacs *et al.*¹ The method differed from the original technique by using antihuman prolactin antiserum (Mercia Brocades) at a dilution of 1/1000 (diluent 0.15 M phosphate buffered saline, pH 7.2) at 4°C for 1 h on material obtained from surgical biopsies. Before staining, the natural endogenous peroxidase activity was blocked using the modified technique that Slocombe *et al.*² used in the demonstration of blood group antigens.

After treatment with 1% osmium, the last stage of the original technique before mounting the section, the sections were gently washed in running tap water for 5 min. They were then washed several times in distilled water. The original technique of Perls³—heated potassium ferrocyanide and 1% hydrochloric acid—was then applied to show the haemosiderin in the section.

The Perls' Prussian blue and the uncounterstained peroxidase serial slides were compared with the peroxidase section counterstained with Perls' Prussian blue using a Zeiss comparison bridge mounted on two Zeiss Standard 18 microscopes. (Carl Zeiss (Oberkochen) Limited)