Technical methods

Use of 3,3′,5,5′-tetramethylbenzidine (TMB) in the identification of erythroid colonies

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Benzidine has been used in the detection of blood since the beginning of the century. Since benzidine is a carcinogen[1] and its future availability is threatened, a safer substitute is required. In 1974 the synthesis of 3,3′,5,5′-tetramethylbenzidine (TMB) was reported and its possible use in the detection of blood was suggested.[2] Since then, it has been tested in the detection of haemoglobin in polyacrylamide gels[3] and as a presumptive test for blood.[4] The benzidine-H_2O_2 stain[5] is used as a definitive test for erythroid colonies in *in vitro* erythropoiesis, the degree of benzidine positivity indicating the haemoglobinisation and hence maturity of the colonies. Substitution of TMB for benzidine in this stain has advantages over benzidine in terms of specificity, safety, and availability.

Material and methods

Erythrocytic colonies were cultured from 4-week-old B alb/C mice in the plasma clot system of McLeod *et al.*[6] with the following differences: bovine thrombin (Sigma London Chemical Co) at a concentration of 1 U/ml was substituted for Beef Embryo Extract (BEE), and alpha medium (Flow Lab Ltd) was substituted for NCTC-109. Alpha medium was purchased in powder form and prepared according to the manufacturer's instructions. Ribosides and deoxyribosides were added to give a final concentration of 10 mg/l of each, and mercaptoethanol was added to the alpha medium to give a final concentration of 10^{-4} M. Erythropoietin (step 3 (CMRL) Connaught Lab, Willowdale, Ontario, Canada) was added at 2·5 U/ml of culture medium. Cultures were set up in disposable microtitre plates (Nunc microtitre plates 1-67008) and incubated for three days at 37°C in a humidified incubator (Leec automatic CO_2 incubator model GA 3) with 5% CO_2 in air.

FIXATION AND STAINING

Glutaraldehyde fixation was carried out as previously described by McLeod *et al.*[6]

Accepted for publication 20 August 1980

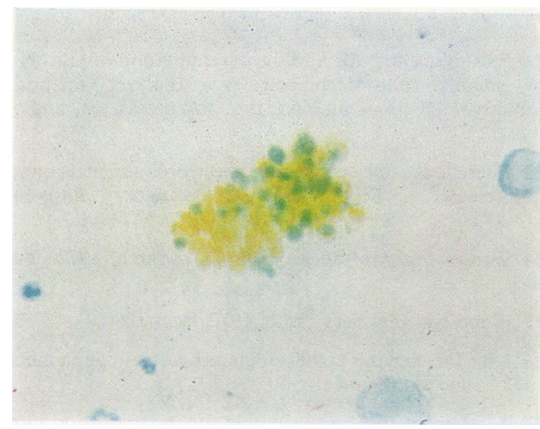
STAINING SOLUTIONS

1 TMB (Sigma London Chemical Co) 0·05% in methanol—can be stored for one month at room temperature.
2 Hydrogen peroxide—0·3% in double distilled deionised water made freshly each day from a stock 3% solution.
3 Resorcinol—1% in Dulbeccos PBS 10 × pH 8·0 (Gibco Biocult Ltd).
4 Giemsa—1 drop/ml of distilled water—made freshly each day.

STAINING PROCEDURES

1 The fixed slides are covered with the resorcinol solution for 5 minutes and then rinsed with distilled water.
2 The slides are flooded with the TMB solution, and the hydrogen peroxide solution is added immediately. The TMB-H_2O_2 solution is left on the slide for 5 minutes and then rinsed with distilled water.

Counter staining is carried out by flushing the slides with the Giemsa solution. The slides are then washed with distilled water and air-dried. In this stain the red cells/haemoglobin are a brown/yellow colour, and the nuclei are green. Counter staining is not necessary unless one wishes to enumerate non-erythroid cells. If counterstained, the cytoplasm of the white cells is as usually seen in a Giemsa-stained slide. The Figure shows the typical appearance of a murine erythroid colony after staining with TMB.



A typical erythroid colony (CFU_E) from a Balb/C mouse after three days. Stained with TMB.

Discussion

The peroxydatic activity of haemoglobin in the presence of hydrogen peroxide catalyses the oxidation of TMB to a coloured product. Haemoglobin peroxidase has been termed a 'pseudoperoxidase'[7] because, unlike true peroxidases which exhibit specificity for phenols, erythrocytes contain a peroxidase (erythrocyte glutathione peroxidase) specific for oxidation of reduced glutathione. This causes the *in vitro* detoxification of hydrogen peroxide. Glutathione peroxidase activity has been found to be associated with a relatively stable, nondialysible, heat-labile, intracellular component which can be separated from haemoglobin by gel filtration and ammonium sulphate precipitation. The pH optimum of glutathione peroxidase has been shown to be pH 8·0 with negligible activities below pH 6·0.[8] Pretreatment of the fixed slides with resorcinol before staining with TMB and hydrogen peroxide inhibits myeloperoxidase staining. This increases specificity of the stain and reduces error in enumeration of erythroid colonies. By omitting counter staining only erythroid colonies and precursors are stained.

This work has been supported by the Pathology Development Foundation, Trinity College, Dublin.

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An automated method for recording the Westergren erythrocyte sedimentation rate

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An automated system for recording the Westergren erythrocyte sedimentation rate (ESR) is described. The method was developed to cater for the frequently occurring but small numbers of late specimens which otherwise delay staff after normal working hours.

Principle

The method involves photography of the ESR tests exactly 1 hour after being set up, in such a way that the photographs are easily and rapidly obtainable when required and the results can be easily read.

Equipment

A Polaroid MP-4 camera with Tominon f4·5, 135 mm lens and Copal shutter is used. This camera employs bellows focusing so that close-up work is easily possible.

A base was constructed to hold the camera and ESR rack at a fixed distance apart, which gives optimum framing of the rack on the film. This base is made mainly of perspex and wood. The circular camera base is held in place by and can rotate in a perspex ring. Using a focusing screen, the camera was aligned as required; then a line was engraved on the camera base and perspex ring. The focus was also marked on the bellows guides, and in this way the camera can be set up rapidly without having to re-align or refocus each time (Fig. 1). The film used is Polaroid Land Type 107C. This is a black-and-white film rated at 3000 ASA (36 Din).

The timing device consists of a shutter-activating system, which uses a simple mains-driven timer (ORMON—Type STPNH, 72 min) which switches on an electric motor after a pre-set time. The motor has a large reduction gear box which gives a shaft speed of 1 revolution per minute. This gives adequate torque from a low-power motor to drive a cam which activates the camera shutter.

The electrical control circuit is simple and as foolproof as possible. There are two parts to it: one is the setting up procedure, and the second is the timer and motor operation.

Accepted for publication 5 November 1980